Genetic control of apoptosis and tumourigenesis in murine models of intestinal neoplasia

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Declaration

I declare that this thesis and the work contained in it is my own, except where specifically acknowledged.

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Acknowledgements

During the course of my PhD, I have been helped by a large number of great people. In fact I have enjoyed my PhD much more than I thought I would. There really are too many people to mention (and I don't want to alter my page count!). After my masters I thought my PhD would only last a few months but I was lucky to live and work with some really good people. Both the labs in Edinburgh and Cardiff have been very supportive. In addition I have some friends that I could have a good rant with. I thank the many people who have had to put up with my near incessant moaning.

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Abstract

Colorectal cancer is one of the most predominant cancers in the Western World. By the age of 70, 1 in 2 people will have a colon tumour. The genetic analysis of both spontaneous and hereditary forms of this disease have greatly added to our understanding of colorectal cancer, with mutations in a range of genes now strongly linked to neoplasia. In recent years, several different transgenic models of colorectal cancer have been generated. Key amongst these are strains mutant for the *Apc* (*Adenamatous Polyposis Coli)* gene and strains mutant for different members of the mismatch repair (MMR) gene family, the majority of which show predisposition to intestinal neoplasia. In this thesis, these transgenic models are used in an attempt to systematically characterise the nature of the genetic control over a series of end points. These include the apoptotic response of enterocytes to cytotoxic agents; the effect of genotype upon clonogenic survival and mutation and ultimately the effect of genoytype upon the development of intestinal neoplasia.

Previously analyses of enterocyte apoptosis had established roles for *p53* and *Msh2* following DNA damage of methylation type. This prompted an analysis in mice deficient for two other members of the MMR family, *Mlh1* and *Pms2*. *Mlh1* and *Pms2* deficient mice were seen to have a significantly reduced apoptotic response to temozolomide, confirming again an association between the MMR family and apoptosis. However, both $Mlh1^{-/-}$ and $Pms2^{-/-}$ mice were found to possess a normal apoptotic response to high levels of the alkylating agent NMNU, even though they are deficient for functional MMR. This unexpected finding dissociates normal mismatch repair from MMR dependent apoptosis and raises fundamental questions about the nature of the death signal following damage of methylation type.

Perturbations to the normal apoptotic response would be predicted to impact upon longer term survival as determined through the microcolony assay. Therefore clonogenic survival was examined using this approach in *Msh2* and *p53* null mice. Despite being necessary for apoptosis for all cytotoxic agents studied, loss of *p53* only led to an increased in survival following cisplatin treatment and not following NMNU or Nitrogen Mustard treatment.

The above data was obtained from morphologically normal tissue. Therefore this analysis was extended to the apoptotic response within intestinal lesions. This displayed that there was both lesion type dependent differences and genotype dependent differences in the apoptotic response. As there were high basal levels of apoptosis in the smallest lesions whilst virtually no apoptosis in adenomas, this highlighted that loss of this apoptotic programme may be crucial to tumour progression.

The interaction between *Msh2* and *p53* in tumourigenesis was also investigated. Both homozygosity and hemizygosity for *p53* were found to dramatically accelerate tumourigenesis on a mismatch (*Msh2*) deficient background. Significantly, the levels of micro-satellite instability (MSI) were highest in tumours which were additionally heterozygous for *p53*. EMSA, Western and immunohistochemisty analysis of these tumours indicated retention of *p53* function in at least a proportion of these tumours. Similar data were obtained from primary cultures, with again increased microsatellite instability and retained *p53* functionality in cultures derived from *p53* heterozygotes. Taken together, this data shows that hemizygosity for *p53* increases microsatellite instability and that, at least in a percentage of tumours, complete loss of *p53* is not a required event. These findings have particular relevance to our understanding of cross talk between *p53* and MMR deficiency in human colorectal disease.

The battery of *in vivo* analyses used throughout this thesis were applied to a new candidate tumour suppressor, *Mbd4*. *Mbd4* deficient mice have no overt phenotype, but fail to mediate normal apoptosis following a wide variety of DNA damage. Following cisplatin treatment, *Mbd4* treatment confers increased clonogenic survival Surprisingly, *Mbd4* mice are not characterised by an in increase in either spontaneous or induced mutation rate, but when crossed to Apc^{Min} mice they accelerate tumour development. These studies demonstrate that *Mbd4* is a central mediator of the response to DNA damage and that it functions as an intestinal tumour suppressor in the mouse.

Finally the ability of aspirin to suppress intestinal neoplasia in murine models of colorectal cancer was examined. Numerous epidemological and animals studies have shown that Non-Steroidal Anti-Inflamatory Drugs (NSAIDS) are associated with

lower risks of colorectal cancer. However studies using aspirin in the $Apc^{Min/+}$ mouse have yielded contrasting results. Here it is shown that aspirin does not reduce tumourigenesis when $Apc^{Min/+}$ mice are put on diet containing aspirin post weaning. However when parents were put on aspirin, a significant suppression of tumourigenesis was observed in the min offspring. In fact there was incomplete penetrance of the $Apc^{Min/+}$ phenotype (40%). To test whether in utero administration of aspirin could also suppress murine models of HNPCC, *Msh2* deficient and ($Apc^{Min/+}$, $Msh2^{-/-}$) deficient mice were examined. In both cases a significant attenuation of tumourigenesis was observed. Taken together this raises the exciting prospect of prophylactic treatment of FAP and HNPCC patients and highlight the power of using transgenic models to investigate intestinal neoplasia.

Abbreviations

5-FU	**5 Fluorouracil**
5mC	**5 methylcytosine**
ACF	**Aberrant Crypt foci**
ADP	**Adenosine diphosphate**
ATase	**O^6-Alkylguanine-DNA-alkyltransferase**
ATP	**Adenosine triphosphate**
APC	***Adenomatous Polyposis Coli***
$Apc^{Min/+}$	**The *Min* mouse**
BeG	**O^6-Benzylguanine**
BER	**Base Excision Repair**
bp	**base pairs**
BrdU	**Bromodeoxyuridine**
CDDP	**Cisplatin**
CIMP	**CpG Island methylator phenotype**
CGH	**Comparative Genomic Hybridisation**
CRC	**Colorectal Cancer**
DAC	**2'-deoxy-5-azacytidine**
DDW	**Deionised Distilled Water**
Dlb-1	***Dolichos biflorus-1***
DMSO	**Dimethyl Sulphoxide**
ES cells	**Embryonic Stem Cells**
FAP	**Familial Adenomatous Polyposis**
gfp	***Green fluorescence protein***
GSK-3β	**Glycogen Synthetase kinase 3β**
H+E	**Heamatoxylin and Eosin**
HN2	**Nitrogen Mustard**
HNPCC	**Hereditary Non-Polyposis Colorectal Cancer**
IDLs	**Insertion Deletion Loops**
i.p.	**intra-peritoneal**

LOH	**Loss of Heterozygosity**
Min	**Multiple intestinal neoplasia**
MBD	**Methyl Binding Domain**
MBD4	**Methyl Binding Domain**
MCR	**Mutation Cluster Region**
MEFs	**Murine Embryonic fibroblasts**
MLH1	***MutL* Homologue 1**
MMR	**Mismatch Repair**
MNNG	**N-Methyl-N'-Nitro-N-nitrosoguanidine**
Mom	**Modifier of Min**
MSH2	***MutS* homologue 2 (human gene)**
MSH2	***MutS* homologue 2 (human protein)**
Msh2	***MutS* homologue 2 (murine gene)**
Msh2	***MutS* homologue 2 (murine protein)**
$Msh2^{+/+}$	**Mouse wild type for *Msh2***
$Msh2^{+/-}$	**Mouse heterozygous for *Msh2***
$Msh2^{-/-}$	**Mouse null, for *Msh2***
MSI	**Microsatellite Instability**
MutSα	**Heterodimer of MSH2 and MSH6**
MutSβ	**Heterodimer of MSH2 and MSH3**
NER	**Nucleotide Excision Repair**
NSAID	**Non-Steroidal Anti Inflammatory Drug**
NMNU	**N'-Methyl-N-nitrosourea**
O^6meG	**O^6-methylguanine**
PBS	**Phosphate Buffered Saline**
PCNA	**Proliferating Cell Nuclear Antigen**
PMS2	**Post meiotic Segregation 2**
RER^+	**Replication Error Positive**
SD	**Standard Deviation**
SDS	**Sodium dodecyl sulphate**
SSBP	**Single Stranded binding protein**
TCR	**Transcription Coupled Repair**
TDG	**Thymine DNA glycosylase**

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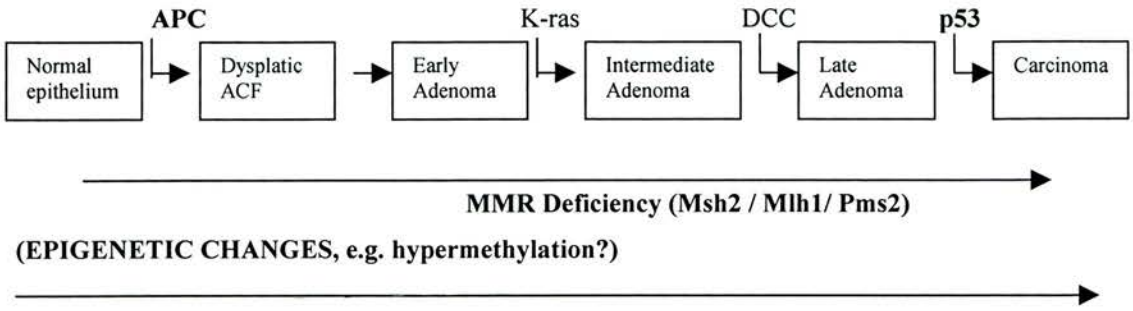
1.0 Introduction

1.1 Colorectal cancer genetics

Cancer is the endpoint of a process whereby normal cells are altered by the cumulative acquisition of genetic changes which confer proliferative, invasive, and metastatic properties upon the cell (see figure 1.1 Vogelstein and Kinzler, 1993). This process can be rapidly accelerated by the loss of systems that safeguard genomic stability resulting in an increased rate of mutagenesis (e.g. loss of mismatch repair) and chromosomal rearrangements (e.g. loss of *p53*) (Hartwell, 1992; Kolodner, 1995). Colorectal cancer is one of the most predominant cancers in the Western World. It is thought that 1 in 2 people over the age of 70 have a colorectal tumour (Vogelstein and Kinzler, 1996). The molecular characterisation of colorectal cancer is the most advanced of all solid malignancies. There are two well characterised pathways (figure 1.1) of colorectal malignancies which probably account for approximately 70% of colorectal tumours. The first of these was proposed by Vogelstein and Kinzler (1996) where there are mutations in *APC/ras/18q* and *P53*, resulting in aneuploid tumours. These account for around 50-60% of colorectal tumours. The second pathway (approximately 20% of tumours) is characterised by loss of mismatch repair activity which results in diploid tumours with high levels of microsatellite instabilty (MSI). These tumours tend to be left sided and have a better prognosis than the aneuploid tumours.

Figure 1.1 Diagram of colorectal cancer progression (Adapted from Vogelstein and Kinzler, 1996). ACF stands for aberrant crypt foci, the first identifiable morphological changes in colorectal neoplasia. Carcinoma refers to invasive tumours.



(The genes studied in this thesis are in **bold**)

This is certainly a simplification of the true situation as many genetic changes have been associated with colorectal cancer so questioning the absolute significance of all these changes. Similarly, regarding the process of neoplasia as a simple series of linear events is likely to be an oversimplification (Ilyas *et al.*, 1999). Recently, a third as yet poorly characterised pathway has been identified wherein tumours are diploid (or near diploid) but do not exhibit MSI. This pathway accounts for up to 30% of all colorectal cancers (Hawkins *et al.*, 2001, Chan *et al.*, 2001).

This thesis focuses upon the use of transgenic models of intestinal neoplasia to investigate the consequences of the loss of function of a number of genes in this pathway (*Apc*, *Msh2*, *p53*) and the implications of this loss for apoptosis, clonogenic survival mutation and tumourigenesis.

1.2 The relevance of apoptosis to tumourigenesis

Apoptosis is a form of programmed cell death. It was originally characterised morphologically, with cells showing plasma and nuclear membrane blebbing, cell shrinkage, chromatin condensation and fragmentation. These changes distinguish

it from necrosis. Necrosis occurs after physical, chemical or osmotic damage to the cells disrupting internal and external membranes of cells and organelles. The cytoplasm of these cells become grossly swollen, and the cell membrane breaks down. This causes the release of denatured proteins and DNA into the intracellular space producing a local inflammatory response. This does not occur after apoptosis where apoptotic fragments are phagocytosed (Kerr *et al.,* 1972, Wyllie *et al.,* 1980).

Apoptosis occurs in normal development and as a genetically controlled response to variety of stimuli e.g. injury / DNA damage. It is the genetic control of the second of these scenarios that this thesis will examine.

There are 2 major areas where apoptosis is hypothesised to play a role in tumourigenesis:

A) Tumor growth and progression

Tumour growth may be thought of as an imbalance between proliferation and apoptosis. Increased cellular proliferation through inappropriate expression of oncogenes (e.g. *c-myc*) and loss of cell cycle arrest (e.g. loss of checkpoint genes) would be predicted to lead to an increase in tumour size. However, if this was accompanied by a comparable increase in the levels of apoptosis then homeostasis would be maintained. In fact, overexpression of the oncogene *c-myc* can also stimulate apoptosis (Evan *et al.,* 1992). Therefore this leads to the prediction that loss of the apoptotic response would be required for the progression of the tumour.

Several groups have attempted to address this simple hypothesis that loss of the apoptotic programmes are relevant to progression of neoplasms. Although it is clear that growth rate of the tumour is not a simple reflection of proliferation (Wyllie, 1985), this hypothesis has not proved the easiest to test. Bedi *et al.,* (1995) showed that during progression of colorectal cancer from normal epithelium to carcinoma there is a reduction in the levels of apoptosis, suggesting

that loss of apoptosis is important to such progression. However when Faezli *et al.,* (1997) investigated the transition from human colonic adenoma to adenocarcinoma, no difference in basal levels of apoptosis was observed. It is possible that by this stage of colorectal tumourigenesis, there has already been a reduction in the levels of apoptosis (see chapter 9). One of the difficulties for these studies investigating basal levels of apoptosis is that are relatively low (0.5-1% of cells in the adenomas that Faezli *et al.,* 1997 studied). In addition, *in vivo* studies only provide a snapshot of the apoptosis that is occurring. It has previously been shown that small differences in apoptosis can actually translate into significant cell loss over an extended period (Vogelstein and Kinzler, 1996). However such differences may often be too subtle to detect.

Many genes that are lost in tumourigenesis e.g. *P53* and *MSH2* and which have been shown to be important in the induction of apoptosis, have also been ascribed roles in cell cycle arrest and repair. This again makes it difficult to assess the importance of apoptosis in isolation. Therefore despite it being widely accepted that loss of apoptosis is important for tumour progression, there is still little direct evidence for this notion.

B) Apoptosis and Chemoresistance

Loss of the apoptotic programme may also be related to gain of resistance to chemotherapy in tumours. Many of the agents used to treat tumours have been shown to induce apoptosis e.g. gamma irradiation, 5-Flurouracil (5-FU), doxorubicin, cisplatin. (Clarke *et al.,* 1997, Bearss *et al., 2*000). Trying to ascertain the significance of this apoptosis is again difficult. A large number of these cytotoxic agents produce *p53* dependent apoptosis, thus loss of *p53* in a high percentage of tumours correlates well with a model where loss of apoptosis produces resistance to therapy (Lowe *et al.,* 1993). However *p53* deficiency is also associated with loss of cell cycle arrest and senescence and so chemoresistance may not necessarily arise due to loss of the apoptotic response.

Probably the best study linking apoptosis to chemoresistance was performed in murine primary lymphoma *in vivo*. Schmitt *et al.,* (2000) demonstrated that overexpression of *Bcl-2* (a potent suppressor of apoptosis though not thought to be involved in arrest) resulted in multi-drug resistance. Interestingly *Bcl-2* had no effect on long term clonogenic survival when these tumours were then cultured *in vitro*. This highlights the difficulties of translating *in vivo* responses *in vitro*.

In fact it is much easier to find instances where there is no link between apoptosis and chemoresistance, than those proving such a link. Bearss *et al.,* (2000) showed that in trangenic MMTV-*myc* and *MMTV-ras/ myc* mammary and salivary tumours, apoptosis was not important in the response to paciltaxel and doxorubicin. Normally these agents induce apoptosis, however in the tumours there was no induction of apoptosis (prior to selection for resistance). Instead the drugs induced cell cycle arrest. However they did show that the tumours with higher basal levels of apoptosis (from biopsy), were more likely to undergo regression.

This thesis investigates both of these questions (the significance of apoptosis and the genetic control of apoptosis post-cytotoxic agents) using murine models of intestinal neoplasia.

1.3 Hereditary colorectal cancer and murine models of intestinal neoplasia

The understanding of the genetic basis of colorectal cancer has been greatly aided by the study of hereditary early onset colorectal cancer. These are thought to account for approximately 15% of all colorectal cancers (Houltson *et al.,* 1992). The two most common of these are FAP (Familial Adenomatous Polyposis) and HNPCC (Hereditary Non-Polyposis Colorectal Cancer).

1.3.1 Familial adenamatous polyposis (FAP)

FAP is an autosomal dominant disease that afflicts approximately 1 in 7000 people (Vogelstein and Kinzler, 1996). The disease is characterised by the development of 100's of adenomatous polyps in the colorectum. This normally occurs during the teenage years and by the age of 25, 80% of patients will have developed cancer. It is thought that these invasive adenocarcinomas arise from the adenomatous polyps. Other intestinal lesions that can be developed by FAP patients include gastric fundic gland polyposis (50%), duodenal adenomas and gastric, pancreatic, bilary or distal small intestinal lesions (Damjanov *et al.,* 1996). Lesions are not confined to the intestine, other tumours incude ostemoas, desmoid tumours and exostoses of long bones.

Patients with FAP have a germline mutation in one copy of the *APC* (adenamatous polyposis coli) gene (Groden *et al.,* 1991, Kinzler *et al.,* 1991). In most cases a further somatic mutation of the remaining allele then occurs leading to the development of adenomas. *APC* is also mutated in the majority of sporadic colorectal cancers (60-80% of tumours) (Rowen *et al.,* 1999, Ilyas *et al.,* 2000). Up to 20 % of sporadic cancers are thought to have lost *APC* function through hypermethylation of its promoter (Toyota *et al.,* 2000). Due to the high frequency of mutations in the *APC* gene in colorectal cancer, it is thought to be a very early change to occur in colorectal neoplasia acting as a cellular "gatekeeper" (Vogelstein and Kinzler, 1996).

APC is a very large protein of 2483 amino acids which interacts with a large number of proteins including: β-catenin, GSK3-β, E-Cadheran, EB1, HDLG (human homologue to disc large gene in drosophila) protein, Protein Phosphatase 2A and axin. Thus for some time its precise mode of action as a tumour suppressor protein has remained unclear (for a recent reviews see Beinz 1999, Polakis 2000). However recently its prime postulated role for tumour suppression is through its involvement in Wnt Signalling (discussed in more detail later) (Morin *et al.,* 1997).

1.3.2 The Min mouse

The *Apc*$^{Min/+}$ (Multiple intestinal neoplasia) mouse was generated through random germline mutagenesis following exposure to ENU (ethylnitrosourea) (Moser *et al.,* 1990, Su *et al.,* 1992). Mice were identified as they had anaemia (displayed by their white feet) and when dissected were found to be moribund with intestinal polyps. Hence the name Multiple Intestinal Neoplasia. It was later discovered the reason why the mice developed polyps was due to inactivating mutations at the Apc locus. The *Apc*$^{Min/+}$ mouse is heterozygous for a nonsense allele at codon 850 of the *Apc* gene (Su *et al.,* 1992). Like FAP, intestinal neoplasia was inherited in a dominant manner. Unlike FAP, the majority of tumours were in the small intestine. However by changing the diet to a more westernised one and by altering the genetic background of the mice, larger numbers of colorectal polyps occur (Bilger *et al.,* 1996). In fact, genetic background can radically alter the *Apc*$^{Min/+}$ phenotype. One of the most resistant backgrounds is the AKR strain of mice. A C57BL/6 *Apc*$^{Min/+}$ mouse develops approximately 30 adenomas at death, compared to only 6 in a mixed AKR x C57BL/6 strain (Dietrich *et al.,* 1993). Using these strain differences, one of the most powerful modifiers of *Apc*$^{Min/+}$ was mapped between D4Mit54 and D4Mit284. This is called *Mom1*^{R} (Modifier of Min) and the most likely candidate gene in this region is secretory phospholipase 2 group 2 *plag2a*, which is expressed mainly in intestinal crypts (Gould *et al.,* 1996).

When polyps from *Apc*$^{Min/+}$ mice on a C57BL/6 background were examined for loss of the remaining copy of *Apc*$^{+}$ all tumours showed loss (Luonga *et al.,* 1994, Levy *et al.,* 1995). However once again there is a background specific effect on loss of heterozygosity (LOH), for example in tumours from AKR mice only 63% showed LOH (Shoemaker *et al.,* 1998).

In contrast to FAP, a small percent of *Apc*$^{Min/+}$ mice develop mammary adenomas (akanthomas) (Moser *et al.,* 1994). Recently Japanese studies have shown a

number of sporadic mammary tumours have mutations in the *Apc* gene (Furuuchi *et al.,* 2000).

Mice homozygous for the $Apc^{Min/Min}$ mutation die in utero (Moser *et al.,* 1990). Other mutations in the *Apc* gene have been made, for example Fodde *et al.,* (1994) generated an truncation in the A*pc* gene at amino acid 716. This again resulted in intestinal neoplasia in the heterozygote and embryonic lethality at day 8 in the homozygote.

To overcome the problem of embryonic lethality conditional mutants for *Apc* have been made using the cre-lox P system. Shibata *et al.,* (1997) made an *Apc* mutant, which lost a region of exon 14 of the *Apc* gene and induced a frameshift mutation in *Apc* upon infection of a Cre recombinase expressing adenovirus. This mutation was also shown to predispose to intestinal neoplasia, as within 4 weeks mice of infection of the colorectal region, adenomas developed.

Therefore given some species variation in phenotype, the $Apc^{Min/+}$ mice do appear a good model of human FAP.

1.3.3: Hereditary Non-Polyposis Colorectal Cancer (HNPCC)

Hereditary Non-Polyposis Colorectal Cancer is an autosomal dominant disease (HNPCC) that accounts for approximately 3% of all colorectal cancers. Patients have an early onset of colorectal and uterine cancers. They also have increased numbers of gastric, upper urinary tract and ovarian malignancies. Patients are designated as HNPCC patients if they conform to the Amsterdam critea (Vasen *et al.,* 1992).

1. Three or more relatives with histologically provable colorectal cancer, one of whom is a first degree relative.
2. At least 2 successive generations affected.
3. FAP excluded
4. One of more of the cancers must have developed before 50 years of age.

Although HNPCC has been recognised for many years, the molecular basis has only recently been characterised. Previously two major critea had been used to define it: linkage of the disease to 2p16 or 3q15 and microsatellite instability (MSI) (Lynch *et al.,* 1991,1993).

2p16 or 3q15 are now known to be the mismatch repair genes *MSH2* and *MLH1* indicating that the HNPCC is caused primarily by a defect in mismatch repair (MMR) (Lui *et al.,* 1996). This also explains why HNPCC tumours exhibit Microsatellite Instability (MSI)/Replication Error (RER+) as MMR is important in repairing microsatellite instability. Microsatellites are small repeats of DNA of 1-5 base pairs that are repeated from 15-35 times. When these are replicated by DNA polymerase often a few of these repeats can be inserted or deleted. These produce insertion/deletion loops (IDL's) that are recognised and corrected by the MMR machinery. Therefore when MMR is lost, MSI ensues (Modrich and Lauhe, 1996). A more detailed overview of MMR will be given later.

Mutations in 5 MMR genes have been associated with colorectal cancer *MSH2*, *MLH1*, *PMS1*, *PMS2* and *MSH6*. Mutations in *MSH6*, *PMS1* and *PMS2* are very rare in HNPCC (Wheeler *et al.,* 2000). *MSH6* deficient tumours do not exhibit MSI and were often overlooked for scoring as HNPCC (Akiyama *et al.,* 1999).

13-15% of other sporadic colorectal cancer exhibit MSI. Many of these tumours have been characterised as having either *MSH2* or *MLH1* mutations. For example in a Scottish cohort 4/7 sporadic colorectal cancers had a *MSH2* mutation (Bubb *et al* 1996). However a large number of sporadic colorectal cancer despite showing MSI, had no identifiable MMR mutation. Recently, it has been shown that in up to 70% of these sporadic colorectal cancer there is indeed no mutation, yet there is hypermethylation of the *MLH1* promoter (Veigl *et al.,* 1998, Herman *et al.,* 1998). Biallelic methylation of the *MLH1* promoter blocks transcription and therefore no MLH1 protein is produced. Further

discussion of the relevance of methylation to colorectal cancer will be given later.

There are also slightly lower levels of *APC* mutations in MSI Colorectal Cancer (CRC) than microsatellite stable colorectal cancers. However there are higher levels of the β-Catenin oncogenic activating mutation (CTNNb1) in MSI CRC indicating that inappropriate Wnt signalling is still necessary for all of these colorectal cancers (Polakis, 2000).

1.3.4 Murine Models of loss of the MMR genes

At present six MMR deficient murine strains have been produced: *Msh2* (*MutS* homolgue 2), *Msh3*, *Msh6*, *Mlh1* (Mut L homologue), *Pms1* (post-meoitic separation) and *Pms2* (for a review see Buermeyer *et al.,* 1999a). Of all these mutants strains, only $Msh3^{-/-}$ and $Pms1^{-/-}$ mice do not have a spontaneous tumour predisposition (Prolla *et al.,* 1998). However *Msh3* deficiency accelerates tumourigenesis on a $Msh6^{-/-}$ background (De wind *et al.,* 1999, Edelmann *et al.,* 1996, 2000). In fact ($Msh3^{-/-}$, $Msh6^{-/-}$) mice appear equivalent to the single $Msh2^{-/-}$ mice.

The spectrum of tumours developed in the MMR deficient mice differs from the $Apc^{Min/+}$ mouse. MMR deficient mice predominantly present with tumours of lymphoid origin (De Wind *et al.,* 1995, Prolla *et al.,* 1998). This difference between HNPCC patients and MMR deficient mice is thought to reflect a species specific difference in spontaneous tumour predisposition: lymphoid tumours occur relatively frequently whilst epithelial tumours are relatively rare in mice (Bradley 1996).

However in both $Msh2^{-/-}$ and $Mlh1^{-/-}$ mice (though not $Pms2^{-/-}$ mice), intestinal neoplasia is observed (Reitmair *et al.,* 1996a, Prolla *et al.,* 1998). Approximately one half of $Mlh1^{-/-}$ mice develop intestinal adenocarcinoma whilst 70% of $Msh2^{-/-}$ mice that survive past 6 months (20% of total) develop

intestinal neoplasia. These older *Msh2*$^{-/-}$ mice also develop extra-intestinal tumours (such as skin neoplasia and uterine tumours) of a comparable spectrum to HNPCC patients (Bradley, 1996). To overcome the strong lymphoma predisposition of the *Msh2*$^{-/-}$ mice, immune compromised (*Tap1*$^{-/-}$, *Msh2*$^{-/-}$) mice were produced. These mice all died from HNPCC-like tumours (De Wind *et al* 1998). Therefore arguing that MMR deficient mice reasonably model human HNPCC. Intestinal neoplasia in the MMR deficient mice is also associated with loss of *Apc*, again arguing the importance of inappropriate *Wnt* signalling for colorectal cancer, with MMR accelerating loss of the *Apc* locus (Reitmair *et al.,* 1996a, Prolla *et al.,* 1998).

One of the powers of transgenics is that by inter-crossing knockouts, one gains insights into pathways and mechanisms e.g. the doubly mutant (*Msh3*$^{-/-}$, *Msh6*$^{-/-}$) mice are equivalent to the single *Msh2*$^{-/-}$ mice. Thus far, *Msh2*$^{-/-}$, *Mlh1*$^{-/-}$ and *Pms2*$^{-/-}$ mice have been crossed onto *Apc*$^{Min/+}$ background. All of these mice show accelerated intestinal neoplasia, even the *Pms2*$^{-/-}$ mice. (Reitmair *et al.,* 1996b, Baker *et al.,* 1998, Shoemaker *et al.,* 2000). Adenomas were shown to have lost expression of *Apc* although the frequency of LOH (loss of heterozygosity) was significantly reduced (Approximately 20% in the *Min Mlh1* -/- tumours). This is consistent with *Apc* being lost in a mismatch repair dependent manner. Taken together these studies indicate that the rate-limiting step for intestinal neoplasia in a MMR deficient background is loss of *Apc*.

In MMR deficient mice, two hits at the *Apc* locus are required for adenoma formation. As MMR deficient mice are prone to lymphoma, it is possible that many mice die of lymphoma prior to development of gut tumours. On an *Apc*$^{Min/+}$ background only one hit is needed, thus animals develop intestinal neoplasia prior development of lymphoma.

In summary, MMR deficient mice model HNPCC neoplasia relatively accurately. HNPCC patients are germline heterozgotes for the MMR mutations. However in the mouse both *Msh2* and *Mlh1* hemizygotes do not develop

intestinal neoplasia (De Wind *et al.,* 1998). In fact when survival data were compared between heterozygotes and wild type mice, there was no difference in survival. However the *Msh2* heterozygotes have more tumours than wild mice although there were no intestinal neoplasia. Only 1/71 tumours showed loss of heterozygosity at the *Msh2* locus in a *Msh2* specific assay PCR. Obviously this could miss many possible mutations, however the only tumour to show MSI was the one that showed LOH at the *Msh2* locus (De Wind *et al.,* 1998). This indicates that Msh2 heterozygosity could alone predispose to tumourigenesis (this will be discussed in more detail later).

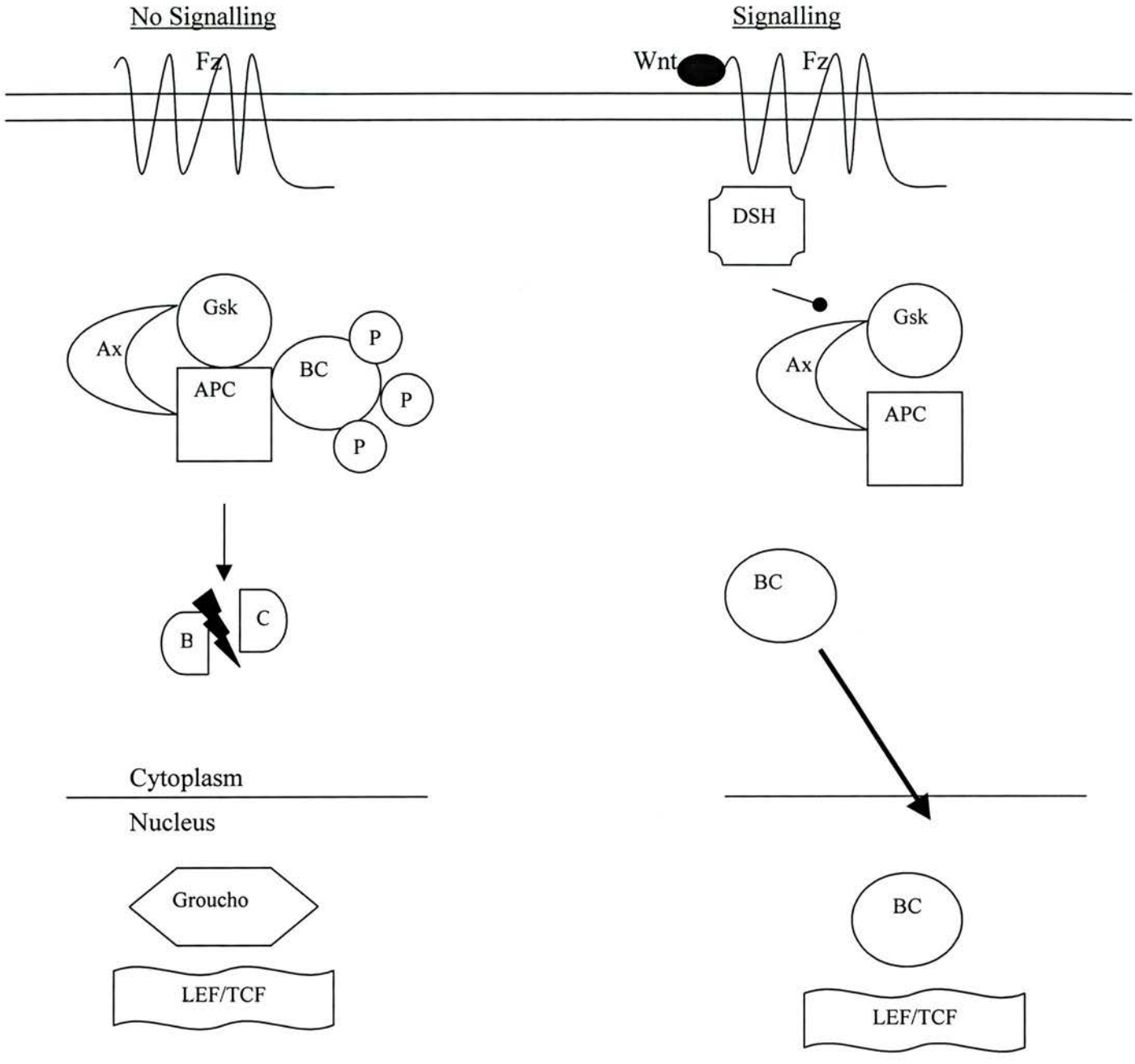
However heterozygosity for *Msh2* did not accelerate intestinal neoplasia on an $Apc^{Min/+}$ background (De wind *et al.,* 1998). Thus despite the possibility of a gene dosage effect of *Msh2* (see chapter 7), in terms of modelling HNPCC, mice completely deficient in MMR appear a closer representation.

1.4 Molecular basis of colorectal cancer

Colorectal cancer is one of the most clearly understood cancers in terms of molecular changes. For some time, several different patterns of genetic linkages have been established for colorectal cancer and recently this has led to the identification of genes mutated in colorectal cancer (Vogelstein and Kinzler 1996, Lengauer *et al.,* 1997).

1.4.1 Apc and Wnt Signalling

The Wnt signalling pathway was first discovered in Drosophila, where it mediates anteroposterior patterning in the segments of the fly embryo (Wieschaus and Riggleman, 1987, Nusse, 1997). In vertebrates the Wnt pathway is important for the induction of the dorsoventral and the anteroposterior embryonic axes. In fact the discovery of *Wnt1* as a proto-oncogene predated the discovery of *wingless* in Drosophila (Nusse, and Varmus, 1982). However it was the elucidation of this pathway that forms the basis of our understanding of the role that *Wnt* signalling plays in cancer.



No transcription

Transcription

Key: BC, β-catenin; Fz, frizzled; Gsk, GSK 3β; DSH, Dishevelled. :

P , Phosphorylation; Ax, Axin.

Figure 1.2: Wn*t* signalling pahtway adapted from Beinz 1999.
This diagram gives a simplified picture of Wnt Signalling. In the absence of a Wnt Signal, β-catenin is phosphorylated targeting it for degradation by the proteasome pathway (Orford *et al.,* 1997). In these circumstances Lef/Tcf transcription is repressed via interactions with Groucho co-repressors.

Following a Wnt signal, Dishevelled is activated by Frizzled. Dishevelled then binds the axin-Apc-GSK3β-β-catenin complex inhibiting the phosphorylation *of* β-catenin. This prevents its being targeted for degradation. β-catenin then translocates to the nucleus where its binds Tcf4, stimulating transcription from *Wnt* target genes. When *Apc* is mutated, *β-catenin* cannot be phosphorylated and therefore transloactes to the nucleus and stimulates transcription.

The Drosophila wingless pathways forms the basis of our understanding of the vertebrate *Wnt* signalling. The pathway seems highly conserved between the *Drosophila* and vertebrates. As expected vertebrate Wnt signalling is more complex with a plethora of Wnt ligands (at least 16) and a large number of *Frizzled* receptors (at least 11) (Polakis, 2000). In response to a Wnt signal in vertebrates, frizzled is activated and transduces this signal to Dsh. Dsh through its interaction with *axin* disrupts the quaternary axin, Apc, Glycogen Synthetase kinase 3β (GSK-3β) and β-catenin complex. This prevents GSK-3β from phosphorylating β-catenin and targeting it for degradation, thus free β-catenin translocates to the nucleus where its stimulates Lef-Tcf transcription (Hart *et al.,* 1998, Beinz, 1999, Polakis, 2000). The Drosophila homologues of *Dsh*, *GSK-3β*, *β-catenin* are *dishevelled, zeste-white 3 kinase* and *armadillo* respectively.

The significance for tumourigenesis is that inappropriate Wnt signalling (such as that caused by a loss of function *Apc* mutation or dominant phosophorylation resistance *β-catenin* mutation) will cause activation of this *Lef-Tcf* transcription. An ever increasing number of target genes for *Wnt* signalling have recently been discovered. These include the oncogenes: *c-myc*, *AP-1*, *Cyclin D1, Wisp1*(He *et al.,* 1998, Mann *et al.,* 1999, Testu and McCormick, 1999, Shtutman *et al.,* 1999, Xu *et al.,* 2000). Therefore this leads to the very attractive hypothesis that following inappropriate Wnt signalling (e.g. loss of *Apc*), transcription of these oncogenes would then cause dysregulated cell proliferation. Thus explaining, the role of *Apc* as a 'gatekeeper' protecting against this dysregulated growth (Vogelstein and Kinzler, 1996).

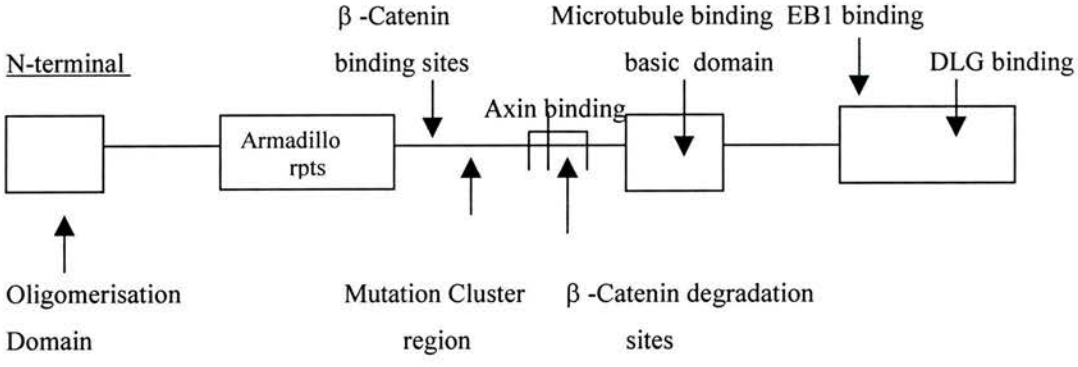
Other genes stimulated by *β-catenin* include *Tcf4* and *Tcf1*, which are both involved in *Tcf/Lef* transcription. *Tcf1* is a negative regulator of *Tcf/Lef* transcription through its interaction with *groucho* co-repressors (Roose *et al.,* 1998). This role as a negative regulator was proved *in vivo* through crossing the $Tcf1^{-/-}$ mice to $Apc^{Min/+}$ mice. Here ($Apc^{Min/+}$, $Tcf1^{-/-}$) mice showed a 10 fold increase in the number of intestinal neoplasms. Thus when *Tcf1* is lost, this additional check on *Wnt* signalling is lost and tumourigenesis is more rapid

(Roose *et al.,* 1999). This experiment also highlights that probably the key role for *Apc* and *β-catenin* in tumourigeneis is through their role in Wnt signalling. However both Apc and β-catenin are large proteins with a number of other functions and interactions which could also play a role in tumourigenesis.

1.4.2 Other Interactions of Apc and β-catenin

1.4.2.1 Apc

Figure 1.3 Structure of Apc (adapted from Polakis 1999) (not to scale). The Apc protein has a number of binding sites in addition to those involved those protein involved in Wnt signalling (β-catenin and axin).



As can been seen from the structure of Apc, in addition to its ability to bind β-catenin, there are also sites for binding microtubules and the proteins EB1 and DLG (Drosophila disc large). The mutation cluster region (MCR) is within the central portion of the protein. Mutations in this region produce truncated proteins that still have the β-catenin binding sites though they would have lost the β-catenin degradation/regulatory sites and the axin binding sites, preventing the formation of the quaternary complex (Polakis, 2000). In addition, microtubule binding, EB1 binding and DLG1 binding would also be lost. Therefore this argues that loss of APC could have additional affects independent of Wnt signalling.

Both *in vivo* and *in vitro* studies have indicated that *Apc* may also interact with the cellular cytoskeleton. In both $Apc^{Min/+}$ and $Apc^{716/+}$ mice migration of intestinal enterocytes up the crypt axis is affected. There is approximately 25% less enterocyte migration up the crypt-villus axis in $Apc^{Min/+}$ mice (Oshima *et al.*, 1995,1997, Mahmoud *et al.*, 1997). Likewise overexpression of *APC* leads dysregulated migration (Wong *et al.*, 1996). Apc also interacts with microtubules, again highlighting a possible role in the cytoskeleton. Apc in fixed epithelial cells localises to clusters at the end of microtubules (Nathke *et al.*, 1996).

Another potential function of Apc comes from its interaction with EB1. A yeast homologue of EB1 has also been shown to a function at cytokinesis, a cell cycle checkpoint (Mihua *et al.*, 1998). Loss of yeast EB1 abolishes this checkpoint which normally delays cytokinesis until the spindle is properly positioned. EB1 is normally located on the microtubules of the mitotic spindle so may act as a sensor for their allignment. Given the ability of Apc to bind microtubules and EB1, it may also be involved in detecting whether the spindle is correctly alligned in vertebrate cells.

Further evidence of a role for Apc in mitosis comes from its localisation to external faces of kinetochores where microtubules attach to chromosome (Kaplan *et al.*, 2001, Fodde *et al.*, 2001). Therefore Apc may facilitate the attachment of the microtubules to the kinetochores in mitosis. When *Apc* mutants were made lacking the C-terminus microtubule binding domains, cells exhibited errors in chromosomal segregation and a propensity to develop aneuploidy. Furthermore, Ishidate *et al.*, (2000) showed that the interaction between APC-hDLG (human discs large) protein negatively regulates cell cycle progression.

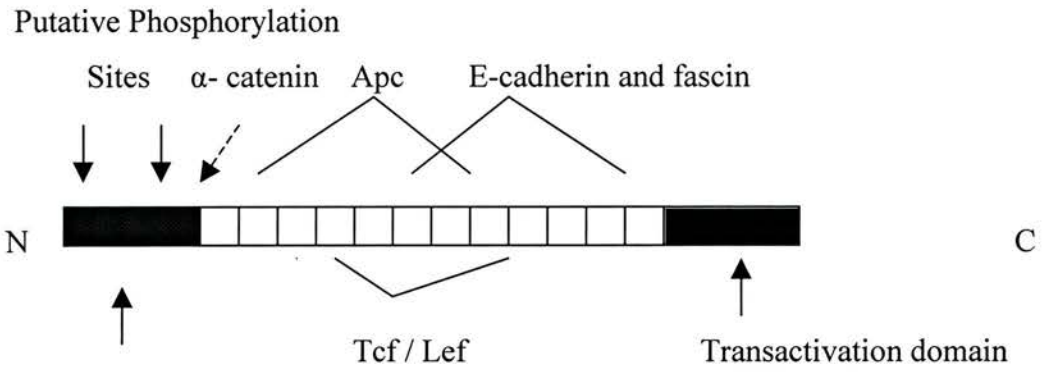
Consistent with all these studies (although most of the mutations in FAP patients occur in the MCR region), several patients have mutations very close to the C-terminus indicating that these potential roles in mitosis and cell cycle checkpoints could also be important in the role of *Apc* in tumour suppression (Pedemente *et*

al., 1998). Although it cannot be discounted that these mutations could affect protein folding and so be the equivalent to inactivating mutations.

In summary, loss of Apc could affect a range of cell cycle checkpoints independently of dysregulated *Wnt* signalling, but it remains unclear how important these functions are to tumour suppression.

1.4.2.2 β -Catenin

Figure 1.4 Structure of β-catenin. Adapted from Wiltert and Nusse (1998). In addition to binding of Apc and Tcf, β-catenin has binding sites for α- catenin, E-Cadherin and fascin.



β-catenin is a protein of 1300 amino acids containing 12 armadillo repeats of approximately 42 amino acids. As can been seen from the figure, Apc, Tcf and E-cadherin bind β-catenin at these arm repeats whilst α-catenin binds at the N-terminus. (Wiltert and Nusse, 1998). In addition to its role in Wnt signalling, β-catenin is also important in cellular adhesion through its interactions with α-catenin and E-cadherin. As these proteins bind to the arm repeats of β-catenin in a mutually exclusive manner with Apc, it indicates that the cellular adhesion function is disparate from Wnt signalling (Hulsken *et al.,* 1994). In epithelial cells, cell-cell adhesion is mediated primarily through epithelial (E) cadherin, a 120 kDa transmembrane glycoprotein localised at adherin junctions (Takeichi, 1991).

β-catenin can bind both α-catenin and E-cadherin at once, thus linking the adherens junction to the cytoskeletal polymer actin. This is essential for the adhesion function of cadherins at the zonula adherens junction. The binding of cadherin-catenin complexes to transmembrane tyrosine kinases and phosphatases allows cells to respond to extracellular signals with rapid and local changes in adhesion.

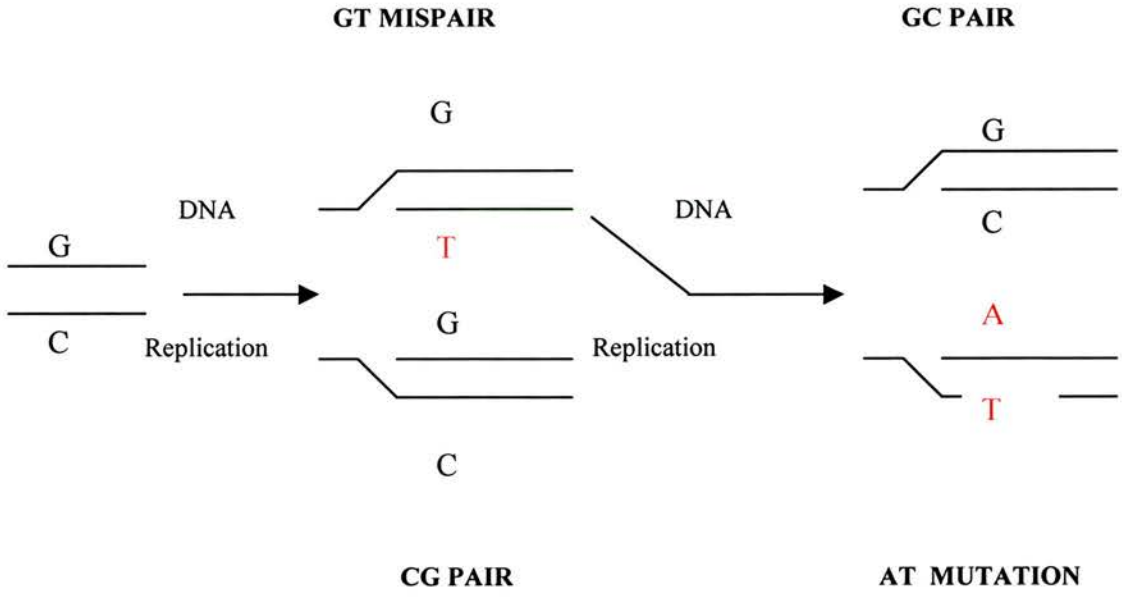
The independence of the cell mobility and Wnt Signalling has been shown by producing mutants of β-catenin. Mutants in the α-catenin binding site can still activate Wnt signalling whilst mutants in the transactivation domain remain efficient in cell adhesion (Orsulic and Peifer, 1996). From the role it mediates in cell adhesion, it is clear that mutations of β-catenin will also lead to altered cell mobility.

In summary, as those colorectal tumours that lack an *APC* mutation, often have a dominant β-*catenin* mutation, this indicates that inappropriate Wnt signalling is the prime mechanism underlying tumourigenesis (Akiyama *et al.,* 2000, Polakis 2000). However both *APC* and β-*catenin* have a number of other potential roles in cell cycle control (e.g. APC with hDLG/spindle control) and cellular adhesion (*B-Catenin*). These other interactions could have quite different implications for the evolution of tumour in terms of both tumour progression and therapy.

1.4.3 Mismatch repair

Originally, most of our knowledge on the basic biology of mismatch repair was derived from unicellular organisms: first *E.coli* and more recently *Sacchromyces cerevisiae* (Modrich, 1994). The conservation of this pathway highlights the importance of mismatch repair to all organisms and not just tumour suppression. The fundamental role of mismatch repair is to correct mispairs produced by DNA polymerase during DNA replication (Modrich and Lauhe, 1996). If these mispairs were left uncorrected it would produce a mutation in the next round of replication (see figure 1.5).

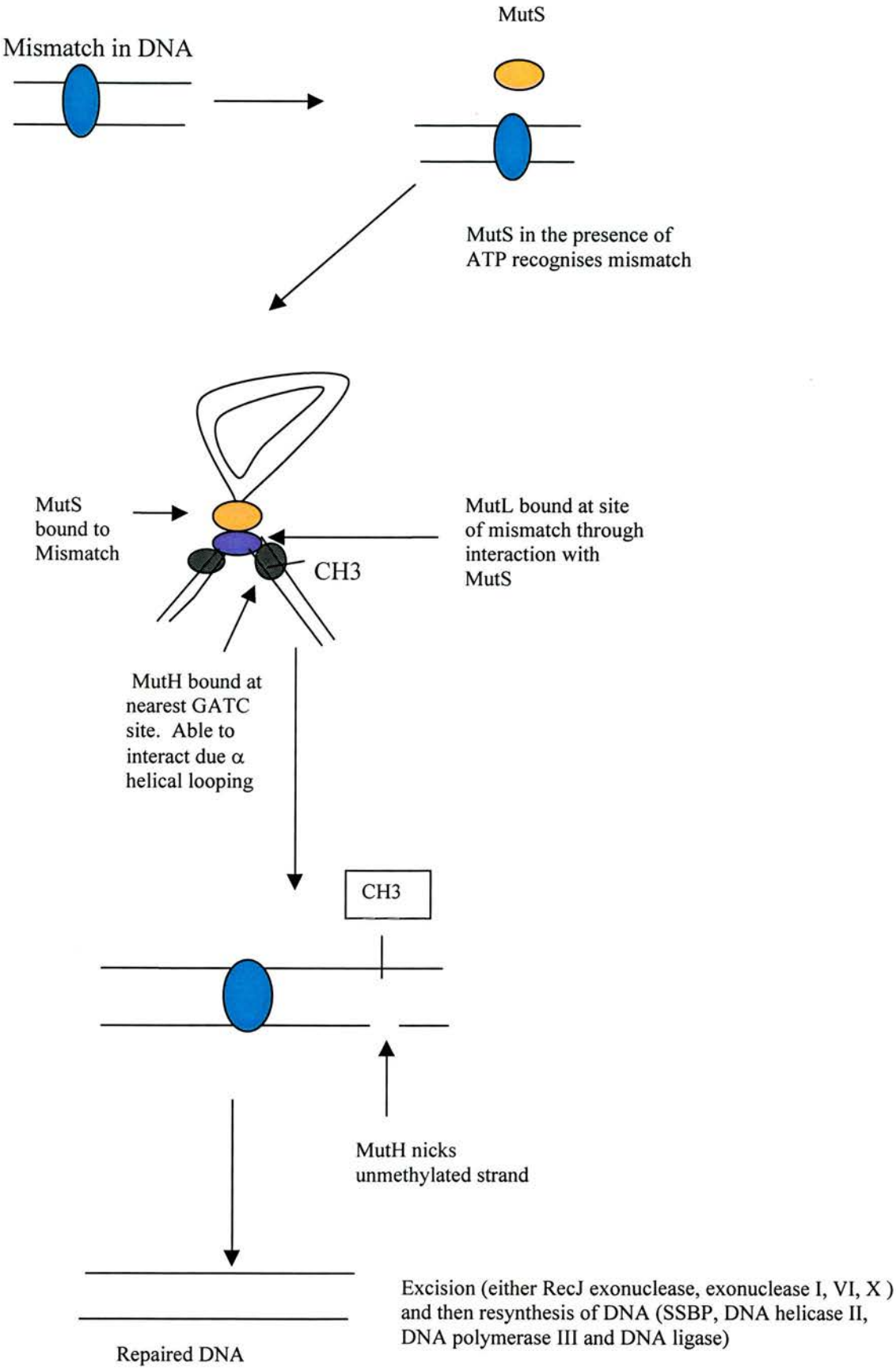
Figure 1.5: The consequence of an unrepaired mismatch (adapted from Modrich 1994).



The best characterised system of mismatch repair is the MutHLS system in *E.coli* (Modrich, 1994, Kolodner, 1995). MutS is the only protein that specifically binds to mismatches of the 10 proteins known to be involved in this mismatch repair pathway (Modrich, 1991). It recognises all mispairs apart from C-C. Out of the 10 proteins only MutS, MutH and MutL are specifically associated with MMR,

the other proteins are more general replication and recombination proteins: Mut U (DNA Helicase II/*UvrD*), Single Stranded Binding Protein (SSBP), RecJ exonuclease, exonuclease I, exonuclease VI, DNA polymerase III and DNA ligase I (Modrich, 1991).

Figure 1.6 Recognition and removal of mismatches by the MutHLS system of *E.coli.*(Adapted from Modrich, 1991)



The binding of MutS to the mispair in the presence of ATP allows the binding of MutL to MutS at the mismatch and allows MutH to bind the nearest GATC site to the mismatch (Modrich, 1991). The ATPase activity of MutS promotes the formation of the α shaped loop of the DNA, allowing the interaction of the proteins at the mismatch with MutH.

MutH alone has a very weak endonuclease activity however in the presence of mismatch, MutS, MutL and ATP, this is activated to produce a single stranded nick in the newly synthesised strand (Welsh *et al.,* 1987). This strand specificity is attained due to the restriction system in *E.coli* (Modrich, 1991). Here the G of the GATC site is methylated by DAM methylase, a hemi-methylase, after replication (Lauhe *et al.,* 1989). Immediately after replication the G of the template strand will be methylated whilst the G of the newly synthesised strand will be unmethylated. Thus MutH nicks the newly synthesised unmethylated strand. MutU is then loaded onto the MutH nick in a MutL dependent manner, unwinding the DNA facillitating exonuclease removal of the nascent strand. 4 exonucleases are involved in MMR : exonuclease I, VII, X and recJ. They apparently work relatively redundantly as it is only when all 4 are knocked out, that MMR is lost (Buermeyer *et al.,* 1999a). The DNA is then resynthesised (Lauhe and Modrich, 1996). This process is bidirectional and depending on the specific exonuclease, DNA can be excised in either a 5' to 3' or 3' to 5' manner from the GATC site. Exonuclease I has a 3' to 5' exonuclease activity whilst exonuclease VII and RecJ has a 5' to 3' activity. (Modrich, 1991).

Although many questions still remain on this MutHLS pathway in *E.coli*: the precise role of MutL, the redundancy of the exonucleases *exoVII* and *RecJ*, the specific stopping of the exonucleases when they have removed the mismatch etc., it is still better characterised than the mismatch repair pathway in eukaryotes (Jirincy, 1994, Fischel and Wilson, 1997).

1.4.3.1 Mismatch Repair in Eukaryotes

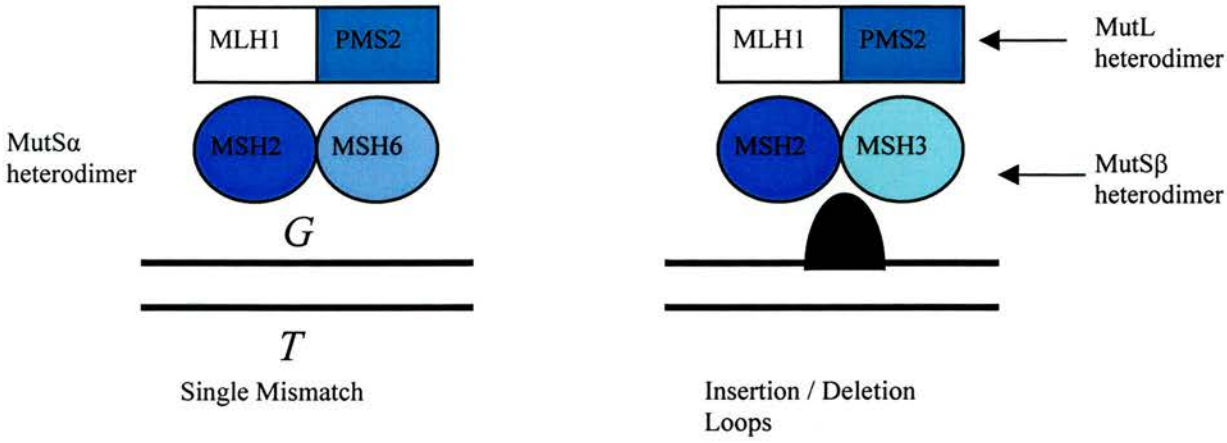
Studies of MMR in eukaryotes have been aided by the conservation of the system between single cell eukaryotes and multicellular eukaryotes. Thus parallel work has been undertaken in yeast, murine models and human cancer cell lines to investigate MMR. The ease of manipulation of the genetics of the yeast *Sacchromyces cerevisiae* has allowed the genetic characterisation of many members of the mammalian MMR pathway (Fishel and Wilson, 1996).

Unsurprisingly, the MMR pathway in eukaryotes appears to be more complex than in *E.coli*. Thus far 6 *MutS* homologues (MSH1-6) have been discovered in yeast and 5 in mammalian cells (MSH2-MSH6) (Fishel and Wilson, 1997). MSH1 has only been shown to be expressed in yeast. Interestingly, it is involved in mitochondrial mismatch repair, raising questions upon possible mechanisms (if any) of mitochondrial MMR in mammals (Chi and Kolodner, 1994).

In both yeast and mammalian cells only three of these MutS homologues have been shown to be involved in mismatch repair: MSH2 (Muts Homologue 2), MSH3 and MSH6 (or GTBP 160) (Modrich and Lauhe, 1996). Much more is known about the action of these proteins than the MutL homologues. The MutL homologues that are thought to be important in MMR are: MLH1 (MutL homologue 1), yeast PMS1 / mammalian PMS2 and possibly yeast PMS2/mammalian Pms1 (Post-meiotic segregration 1 and 2) (Fischel and Wilson, 1997, Prolla *et al.,* 1998). This confusion in the nomenculature is because PMS1 in yeast has the greatest identity to PMS2 in mammalian cells and PMS2 in yeast has the greatest identity to PMS1 in mammals. As most of thesis is concerned with murine models of these genes when I discuss PMS1 and PMS2 this will refer to mammalian PMS1 and PMS2, yPMS1 and yPMS2 refer to the yeast genes. Recently 2 other MutL proteins have been shown to exist in both yeast and mammalian cells : MLH2 and MLH3 however their role in MMR like PMS1 remain obscure (Wang *et al.,* 1999, Lipkin *et al., 2000*). So far no MutH

homologues have been found in eukaryotes nor a mechanism for strand specificity.

Figure 1.7 Proposed model of mismatch recognition in eukaryotes (adapted from Fischel and Wilson, 1997, Alani *et al.,* 1997)



MSH2 acts as a heterodimer with MSH3 or MSH6 in mismatch recognition. At first it was proposed that MSH2 worked alone as a homodimer which recognised mismatches (Jirinchy, 1994). However *in vitro* it recognised mismatches quite weakly compared to the mismatch binding activity of MUTSα that was isolated from yeast (Iccarino *et al.,* 1996). When this complex was isolated first in yeast and then in human cells, it was shown to be a heterodimer of MSH2 and a 160kda protein: MSH6/GTBP 160 (Habraken *et al.,* 1996, Drummond *et al.,* 1995). This extract was shown to then restore MMR in MMR deficient human colorectal cancer cell lines HCT-115 and LoVo (Drummond 1995).

The HCT-115 cell line was shown to have a functional *MSH2* gene and a mutated *MSH6* gene. As expected this cell line shows an inability to bind single mismatches producing an increased mutation rate (mutator phenotype). It also showed instability in its mononucleotide tracts. However microsatellite instability at dinucleotide and trinucleotide tracts; common in yeast and colorectal cancer cell lines with the *MSH2* mutation was not evident (Papadopoulous *et al.,* 1995,

Johnson *et al.,* 1996). This indicated that there was likely to be another protein involved in preventing this microsatellite instability. It was shown eloquently in *S.cerevisaie* that this other protein involved in MMR was the other cloned MutS homologue MSH3 (Johnson *et al.,* 1996, Marischky *et al.,* 1996). In *S.*cerevisiae the *msh3* mutation alone has a very weak mutator phenotype, which is almost indistinguishable from wild type, though its MSI is 40 fold higher. Although *msh6* has a strong mutator phenotype this is still lower than *msh2* mutant and there is no MSI. However the *msh3 msh6* double mutant has a mutation rate and spectrum that is indistinguishable from the *msh2* mutant, indicating that MSH3 and MSH6 interact in a partially redundant manner with MSH2 (Johnson *et al.,* 1996, Marischky *et al.,* 1996).

More recently this interaction has been confirmed in mammalian cells using transgenic mouse knockouts. The combined ($Msh3^{-/-}$, $Msh6^{-/-}$) mouse gave a comparable phenotype to the single $Msh2^{-/-}$ mice (De wind *et al.,* 1999, Edelmann *et al.,* 2000).

The interaction between MSH2 and MSH3 was confirmed *in vitro* when the MSH2-MSH3 heterodimer was purified (MUTSβ complex) (Habraken *et al.,* 1996). As predicted this complex was shown to have a low affinity for single GT mismatches and a high affinity to bind larger insertion/deletion mismatch loops. This produces the model shown above where MSH2 in a heterodimer with MSH6 or MSH3 recognises single mismatches and insertion/deletion loops (IDLs)

Mutations in *Mlh1* and *Pms2* produce a mutator phenotype both *in vitro* and *in vivo* (Prolla *et al.,* 1998, Glaab and Tindell 1997). Therefore it is known that *Mlh1* and *Pms2* are essential for MMR although like the situation in *E.coli*, their precise function is not known. However again it is postulated to act as a 'molecular matchmaker' coupling mismatch recognition to downstream removal of the mismatch. The MLH1-PMS2 in humans and the MLH1-PMS1 heterodimers in yeast have been purified (Li and Modrich, 1995, Habriken *et al.,*

1997). Although this heterodimer is unable to recognise mismatches (as expected from *E.coli*), the heterodimer promoted the binding of MSH2-MSH3 and MSH2-MSH6 to mismatches (Habriken *et al.,* 1997). Another MutL heterodimer MLH1-PMS1 heterodimer has been isolated in human epithelial cells though its function is unclear. (Raschle *et al.,* 1999).

After mismatch recognition, ATP hydrolysis appears to be required in the following steps of the MMR pathway though the actual details of these are still unknown (Alani *et al.,* 1996,1997). Recently, Fishel (1998, 1999) proposed that the MSH (Muts S Homologues) proteins act as a ADP-ATP molecular switch. Thus, on recognition of the mismatch there is ADP to ATP exchange which induces a conformational change of the MSH proteins meaning that they can act as a sliding DNA clamp, which then directly interacts with the downstream MMR machinery (Gradia *et al.,* 1998, Fishel, 1998, 1999). One potential 'effector'of MMR is PCNA (Proliferating Cell Nuclear Antigen). PCNA can bind both the Mutsα (via MSH6) and Mutsβ (via MSH3) as well as MLH1 (Buermeyer *et al.,* 1999a, Flores-Rozas *et al.,* 2000). Flores-Rozas *et al.,* (2000) showed that PCNA is involved in the mispair recognition complex. This interaction with PCNA may provide the basis for strand specificity in mammalian cells. As was said earlier there is no MutH homologue nor restriction system in mammalian cells so questions have remained on strand discrimination. An interaction with PCNA could tether the MMR machinery to the replication proteins during DNA replication, allowing rapid repair of mispairs. Alternatively PCNA binding on DNA post replication could then allow loading of the MMR proteins onto the new synthesised strand allowing it to recognise mispairs. Fishel's 'molecular switch' model proposes that the MMR sliding clamp interacts with PCNA, which is also a sliding clamp causing the polymerase to switch from a forward processivity to a reverse processivity steered by it 3' to 5' exonuclease domain (Fishel, 1998, 1999, Gradia *et al.,* 2000). However this model is still highly speculative. Thus far all that has been shown is the ADP to ATP switch.

Bellacosa *et al.,* (1999) argued that a MLH1 interacting protein MBD4/MED1 could act as functional homologue of MutH for strand discrimination. It is a member of the methyl binding domain (MBD) family of proteins and has been shown to interact with methylated DNA, MLH1 and cause MSI when overexpressed. When examined more carefully *Mbd4* appears to be a mismatch specific thymine glycolase. Thus it was able to remove Thymine or Uracil mispaired with Guanine. However it could only bind to symmetrically methylated sequence or a mismatch in the context of a MeCpG (5 methylcytosine: guanine) (Hendrich and Bird 1998, Heindrich *et al.,* 1999). Given that MeCpG makes up only a very small percentage of the genome, it would be highly unlikely that *Mbd4* could act as a general MutH homologue. (*Mbd4* is discussed at length in chapter 8).

1.4.3.2 Mismatch Repair and Apoptosis: Methylation Resistance.

MMR is thought to be involved in the clearance of lesions in the DNA produced by methylating agents and cisplatin (Modrich and Lauhe 1996, Fischel and Wilson 1997). Thus the loss of MMR genes could be important in acquiring chemoresistance to these agents. The evidence for this includes both biochemical and genetic evidence.

Duckett *et al.,* (1996) showed that human MUTSα specifically bound DNA containing O^6 methylguanine (O^6meG), O^4 methylthymine (O^4meT) and cisplatin induced 1,2 intrastrand crosslinks. Ceccotti *et al* (1996) showed a similar phenomenon in human colorectal cancer cell lines where methylated plasmids (methylated by NMNU producing O^6meG adducts) introduced into cells caused repair synthesis to occur in MMR proficient cells. However in MMR negative cells (e.g. LoVo *MSH2*$^{-/-}$) no repair synthesis was shown to occur.

Originally the majority of the genetic evidence focussed upon human tumour cell lines lacking MMR enzymes (normally *MSH2* or *MLH1)* and exposing them to methylating agents e.g. MNNG, NMNU or temozolomide or other agents such as

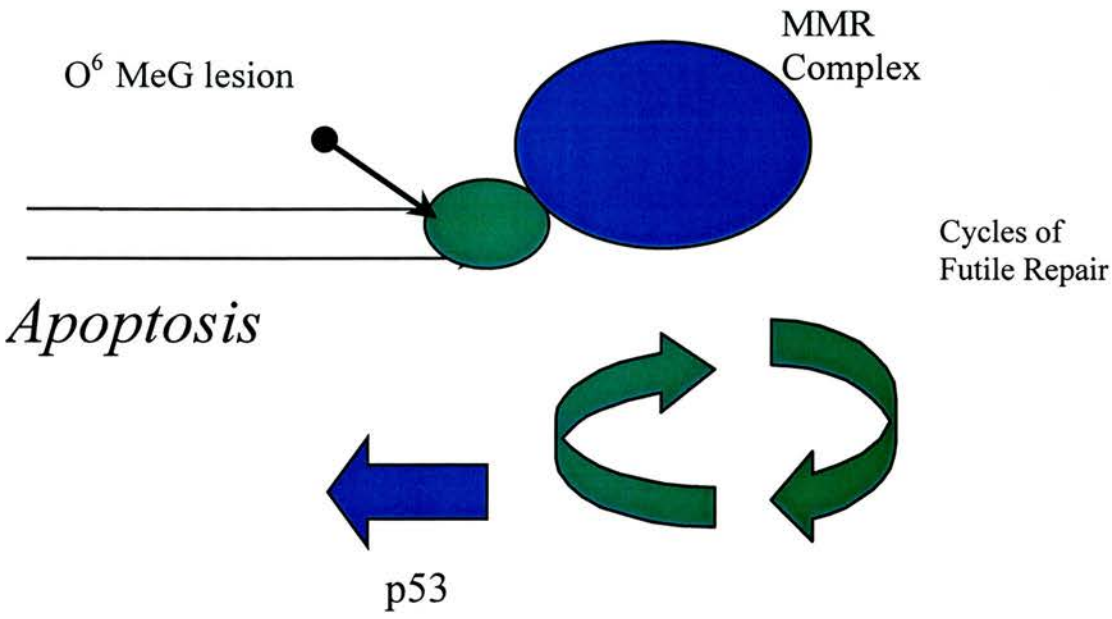
cisplatin. NMNU (N-methyl-N-nitrosourea), MNNG (N-methyl-N'nitro-N-nitrosoguanidine) and temozolomide all produce O^6meG adducts (Pegg 1990, Newlands *et al.,* 1997). These MMR deficient tumours cell lines showed increase resistance and clonogenic survival (Fink *et al.,* 1997).

One of the difficulties of using *in vitro* tumour cell lines is proving that the reason for this resistance is loss of MMR rather than another genetic alteration in the tumour cell line (Fink *et al.* 1997). For example many of the tumour cell lines which lack MMR (e.g. LoVo, HCT-15 and DCD1) have increased levels of ATase (O^6 alkylguanine transferase), an enzyme reponsible for removing O^6 meG adduct *in vivo* (Pegg, 1990). Thus this could be the cause of the methylation resistance. However, Branch *et al.,* (1995) showed that the SW48 colorectal cancer cell line lacking *MSH2* exhibits methylation resistance despite having low levels of Atase, although this still did not provide a conclusive link between MMR and apoptosis.

The relationship between loss of MMR and methylation resistance was established by complementation studies where the mutant cell line had the wild type chromosome (which contained the MMR gene) added back to the cell line. In this instance when the original tumour cell line was compared to the complemented one, most of the methylation resistance was lost (Hawn *et al.,* 1995). Hawn *et al.,* (1995) also showed that complimented cells now entered a G2 arrest, implicating MMR in producing a G2 arrest.

Probably the best evidence of the loss of MMR leading to methylation resistance was from studies using the MMR deficient mice. *Msh2*$^{-/-}$, *Mlh1*$^{-/-}$ and *Msh6*$^{-/-}$ embryonic stem (ES) cells and primary embryonic fibroblasts derived from MMR mice, all show methylation resistance (De Wind *et al.,* 1995,1999, Buermeyer *et al.,* 1999b, Toft *et al.,* 1999). Such primary lines carry the considerable advantage that they will not have been selected for additional genetic change in culture, and so should parallel the genotype of the mutant strain.

Figure 1.8: Replicative Cycling model of MMR dependent apoptosis (adapted from Karran and Bignami, (1991)



The mechanism by which the MMR pathway is invoked in clearance of the damage caused by methylating agents is still quite speculative. Studies on *Msh2* deficient mice and cells have shown that there are reduced levels of apoptosis compared to wild type cells following treatment with methylating agents (Modrich and Lauhe, 1996, Toft *et al* 1999).

The alkylating agents MNNG, temozolomide and MNNG all produce a similar spectrum of lesions (see appendix). However, the lesion that is thought to be important for MMR recognition and apoptosis is the cytoxic lesion O^6meG (methylguanine lesion). The reason for this is that O^6meG causes a misincorporation of a T opposite the G. Thus the MMR machinery will recognise this as a mismatch, excising the newly synthesised T. The replicative cycling hypothesis of MMR dependent apoptosis then suggests that, as the newly synthesised T is removed and not the O^6 meG lesion, another T will be re-inserted again which will be recognised again by the MMR machinery. This produces

cycles of removal and re-synthesis. It is proposed that this continual cycling of the repair machinery causes a signal to induce apoptosis either due to energy depletion or long term presence of single strand breaks (Karran and Bignami, 1991, Fishel and Wilson, 1997, Fink *et al.,* 1997).

However the precise nature of this apoptotic signal is still under debate. Fishel's (1999) molecular switch model argued against this futile cycling and instead a more direct signalling from the MMR complex to the apoptotic machinery (Gradia *et al.,* 2000).

For apoptosis to be basis of the methylation tolerance, this short term decrease in apoptosis needs to translate into longer term (clonogenic) survival. Furthermore, MMR deficiency may translate into an increased mutation rate (compared to wild types) due to long term perpetuation of cells bearing a higher level of DNA damage. Although increased survival and mutation seem two very obvious consequences of failure to engage gene dependent apoptosis, it should be noted that in many instances this is not the case, as has been shown for *p53* (see chapter 6).

Several studies appear to confirm these two predictions for MMR and methylation resistance. Toft *et al.,* (1999) showed that *Msh2* null mice had a reduced level of apoptosis when they were treated with MNNG or temozolomide and that *Msh2*$^{-/-}$ ES cells have increased clonogenic survival when treated with temozolomide.

Two studies have reported that *Msh2*$^{-/-}$ mice have an increased mutation frequencies *in vivo* in response to these cytotoxic drugs. Andrew *et al.,* (1998) showed this using a lacI reporter system (this is described in more detail in 1.4.5.2). After injection with NMNU, *Msh2*$^{-/-}$ mice had significantly higher frequencies than wild type animals. This was significantly higher than differences between untreated *Msh2*$^{-/-}$ mice and wild type animals (Andrew *et al.,* 1998).

One other study has examined the impact of second methylating agent temozolomide on *in vivo* mutation frequency (Toft *et al.,* 1999). This study used the *Dlb-1b* mutation assay (described in more detail in 1.4.5.2 and in Winton *et al.,* 1988). Again the basal mutation frequency was higher (X10) in untreated *Msh2*$^{-/-}$ mice when compared to heterozygotes and wild type animals. Heterozygotes and wild type animals had similar levels of mutation.

When temozolomide was administered a significant increase in mutation rate was observed between *Msh2*$^{-/-}$ mice and wild type and heterozygous mice. These two studies provide clear evidence that *Msh2* is involved in clearance of lesions produced by the methylating agents NMNU and temozolomide. Thus, this increase in survival and mutation correlates well with the loss of the apoptotic response.

As mentioned earlier, MMR deficient cell lines and primary fibroblasts, also lose the G2 arrest in response to these methylating agents (Hawn *et al.,* 1995, Buermeyer *et al.,* 1999b). Thus failure of the cell to arrest could also explain the increased survival and increased mutation. One potential method of obviating the arrest that could be MMR dependent is through replicative bypass around the lesion (Moreland *et al.,* 1999). The basis behind this is that MMR is also involved in preventing illegimate 'homeologous' recombination (De wind *et al.,* 1995, Dudenhöffer *et al.,* 1998). MMR binds to the mispaired recombination intermediates so preventing recombination to occur. The theory of replicative bypass of the lesion is that replication complex becomes stalled at a lesion, producing a G2 arrest. However illegimate recombination around the lesion occurs in cells that lack MMR, causing loss of G2 arrest and permitting proliferation to occur. The best evidence for this comes from studies with cisplatin (see 1.4.3.3). In the case of methylation damage, as MMR both binds the O^6meG lesion and signals apoptosis from it, this appears to be a direct role in damage recognition rather than replicative bypass of the O^6meG lesion (Moreland *et al.,* 1999, Buermeyer *et al.,* 1999a).

1.4.3.3 Mismatch repair and cisplatin resistance

Duckett *et al.,* (1996) showed that human MUTSα bound DNA and cisplatin induced 1,2 G-G intrastrand crosslinks. Unlike the O^6meG lesion which produces the GT mispair, the basis of how MMR recognises this lesion is unclear. However the evidence that MMR deficiency plays a subtle role in contributing to cisplatin resistance is clear.

Cisplatin is often used as a chemotherapeutic in ovarian carcinoma, however drug resistance often follows initial regression of the tumour (Aebi *et al.,* 1997). Strathdee *et al.,* (1999) showed that approximately 12.5% of these tumours demonstrated loss of function of *MLH1* through promoter hypermethylation.

When the MMR complemented ovarian cell line A2780 was examined in respect to cisplatin tolerance, a series of tumour cell lines had around a 2 fold higher tolerance to cisplatin than the MMR complemented cells (Fink *et al.,* 1996,1997). Carboplatin showed similar MMR dependent resistance although other platinated compounds such as oxaliplatin exhibit no such differences in resistance (Fink *et al.,* 1996,1997).

Due to this relatively low increase in resistance, further experiments were carried out to examine the significance of this phenomenon. Fink *et al.,* (1997) did this by selecting resistance to cisplatin both *in vivo* and *in vitro*. This was done *in vitro* by taking HCT116 MMR deficient cells, which expressed *gfp* (*green fluorescence protein*) and culturing these with HCT116 MMR proficient cells that did not. 5% of the cells at the start of the experiment were MMR deficient but 5 days later following a one-hour exposure to cisplatin there were 53% more MMR deficient cells than in the untreated controls.

In vivo selection experiments were performed by implanting wild type and *Msh2* null ES (embryonic Stem) cells subcutaneously into nude mice. The mice were then treated with cisplatin. The wild type tumours shrank in size over the first ten

days. In the *Msh2* null tumours, no shrinkage of tumours was observed, instead there was a growth delay for 4 days. 18 days after the implantation the *Msh2* null tumours had a significantly higher volume (Fink *et al.,* 1997).

One of the most eloquent studies showing that MMR is important in cisplatin resistance was shown using ovarian tumour xenografts lacking MLH1 due to promoter hypermethylation. After addition of 2'-deoxy-5-azacytidine (DAC), a methylation inhibitor, MLH1 was re-expressed. Coincident with MLH1 re-expression, the xenografts were re-sensitised to cisplatin (Plumb *et al.,* 2000).

However this appears to be a rather subtle affect as loss of MMR only confers a 2 fold increase in resistance compared to a 50-100 fold increase in resistance to alkylating agents (Branch *et al.,* 2000). When apoptosis was examined in $Msh2^{-/-}$ mice, there was only a small decrease in the level of apoptosis compared to complete loss of apoptosis in $p53^{-/-}$ mice at the same timepoint (Toft *et al.,* 1999). Branch *et al.,* (2000) showed, in ovarian tumour cell lines, that MMR deficiency was relatively minor when compared to p53 deficiency.

As stated above, MMR may initiate a G2 arrest following cisplatin treatment by preventing replicative bypass of the cisplatin lesion. The best evidence for this was shown using yeast mutants for MMR and DNA recombination. Durant *et al.,* (1999) showed that inactivation of *mlh1* and *msh2* caused resistance to cisplatin. However when they were crossed to recombination deficient *rad52* or *rad1* mutants, the resistance was lost. This indicates that MMR resistance was through a recombination mediated pathway. In a similar study in ovarian cell lines the DNA polymerase inhibitor Aphidicolin was used to inhibit the replicative bypass (Moreland *et al.,* 1999). *In vitro* studies have shown that DNA polymerase δ and ε are able to bypass 1, 2 crosslinks induced by CDDP at replication forks (Hoffmann *et al.,* 1996). The addition of aphidicolin to MMR deficient cells caused re-sensitisation to cisplatin (Moreland *et al.,* 1999).

This replicative bypass model could account for the recent proliferation of DNA damage responses that MMR is associated with. MMR deficiency has been associated with ionising radiation, 6-thioguanine (6-TG), doxorubicin, 5-flurouracil (5-FU), oxidative damage, etoposide and chloroethylating agents (Fink *et al.,* 1997, Durant *et al.,* 1999, Davies *et al.,* 1998, De Reese *et al.,* 1998, Hickman and Samson, 1999). Out of these only 6 –TG would produce a substrate the MMR machinery would recognise directly i.e. a mismatch. Thus it could be that replicative bypass could account for MMR's minor role in resistance to these types of damage.

Another explanation for the growing number of roles of the MMR proteins could be that they are members of a much larger 'repairosome' complex. Recently in HeLa cells a large complex called BASC (BRCA1-associated genome Surveillance complex) containing BRCA-1, MSH2, MSH6, MLH1, ATM, BLM, and the RAD50-MRE11-NBS1 protein complex was isolated (Wang *et al.,* 2000).

As MMR has been shown to be important for apoptosis and cell cycle arrest this has recently led to a debate about what is the primary reason for selection of loss of MMR in colorectal cancer. The previously accepted theory was that a mutator phenotype is required for tumourigenesis, thus loss of MMR provides this (Loeb *et al.,* 1991,1993). However more recently this view has been challenged. Tumours from lacI and lacZ transgenic mice from both *p53* and *Msh2* null tumours do not always show an increased mutational burden compared to wild type tissues (Sands *et al.,* 1996, Andrew *et al.,* 2000). Bodmer and Tomlinson (1999) have argued that the selection benefit for the cell of a mutator phenotype would not take effect immediately so that there would not be no positive selection for it. Instead they argue that selection for loss of MMR is for loss of apoptosis or arrest which would give an immediate selective advantage. However a secondary effect would be to produce a mutator phenotype (Ilyas *et al.,* 1999). As yet the models of somatic evolution of tumours are not yet sufficiently advanced to answer these questions. One problem with this theory is that thus far MMR-dependent apoptosis and arrest has only been displayed after a high level of a

specific drug/ DNA damaging agent. Whether this is relevant in an untreated tumour has not been shown. One study that argues against this is Plumb *et al.*, (2000) which was described earlier. After treatment with DAC, there is re-activation of MLH1 which causes sensitisation to cisplatin treatment. However, when MLH1 was re-expressed in untreated tumours there was no effect on cell growth. Thus if loss of MLH1 equated to loss of apoptosis or cell cycle arrest, one would expect some decrease in cell growth in these untreated tumours after re-expression of MLH1.

1.4.3.4 Other MMR functions

This sections provides a very brief overview of other roles that have been ascribed to the MMR proteins.

1. Meiosis:

The MMR proteins: Mlh1, Pms1, Pms2 and Msh4 have been shown to be important in meiosis. In fact all *Mlh1*$^{-/-}$ mice and male *Pms2*$^{-/-}$ mice are sterile (Edelmann *et al.*, 1996, Bakker *et al.*, 1995.)

2. Somatic Hypermutation.

The MMR proteins Msh2, Mlh1 and Pms2 also play a role in somatic hypermutation. Somatic hypermutation is the process of immunoglobulin (Ig) gene somatic mutation to introduce specific nucleotide changes into the variable region of the Ig during the immune response (Reynauld *et al.*, 2000). Surprisingly loss of MMR causes reduction in hypermutation. In addition the spectrum of mutations in the different MMR mutants also differ. (Reynauld *et al.*, 2000)

3.**Transcription Coupled Repair (TCR) and Nucleotide Excision Repair (NER).**

Functional overlap between MMR and Nucleotide Excision Repair (NER) has been shown on many occasions. Many types of DNA damage e.g. cisplatin lesions are thought to be recognised by both NER and MMR proteins (Reed 1998,

Jordan and Carmo-Fonseca, 2000). NER has also been shown to supress 'homeologous' recombination and be involved in repair of certain mismatches (Sugwara *et al.,* 1997). One type of mismatch that NER recognises is the C-C mismatch not recognised by MMR (Fleck *et al.,* 1999). Some types of repair require both MMR and NER. Kirkpatrick and Petes, (1997) showed that *Msh2* and *Rad1* were both required to repair 26bp DNA loops in *S. cerevisaie*.

Probably the best evidence for an interaction between the MMR and NER genes occurs in Transcription Coupled Repair (TCR). In *E.coli, MutS* and *MutL* mutants selectively abolish rapid repair in the transcribed strand and render the cells moderately sensitive to UV irradiation (Mellon and Champe, 1996). *MSH2* has been shown to physically interact with NER genes R*AD2, RAD10, RAD14* and *RAD25* in yeast (Bertrand *et al.,* 1998). Post UV damage, mutations in *MSH2* increases the UV sensitivity of NER deficient yeast mutants. Tumour cells lines deficient in MMR have also been shown to be deficient in TCR (Mellon *et al.,* 1996). Leadon and Avrutskaya, (1997) showed that $MSH2^{-/-}$ tumour cell lines were deficient in TCR of adducts of UV and oxidative damage (Leadon and Avrutskaya, 1997). Surprisingly *MLH1* mutants were only defective in TCR of UV and not oxidative damage.

However recently it has been shown that, in $Msh2^{-/-}$ mice, the levels of TCR following UV damage was normal so questioning the significance of this *in vivo* (Sonneveld *et al.,* 2001).

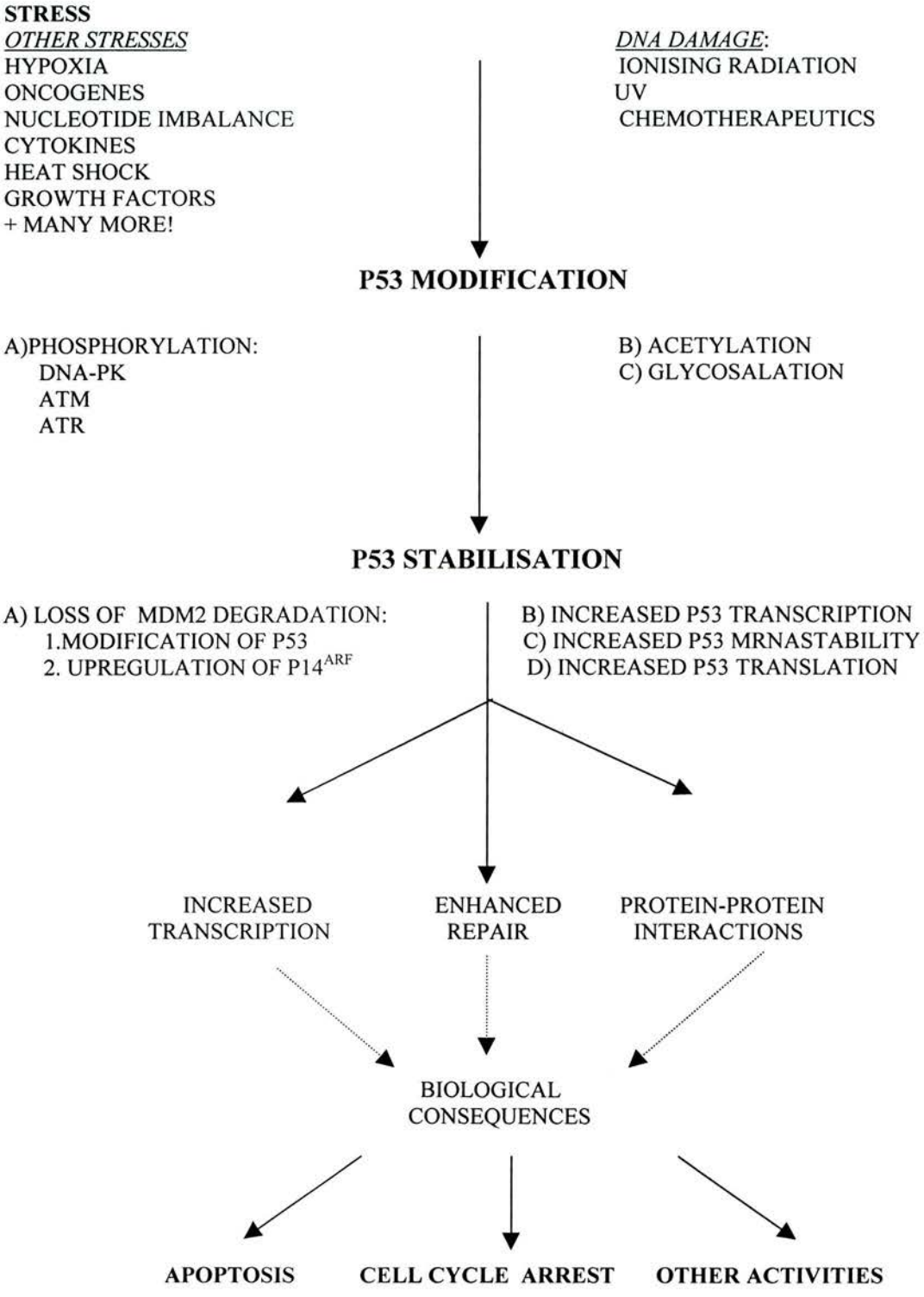
1.4.4. p53 and colorectal cancer

Any search on *P53* soon yields a staggering number of publications (in 1998 alone there were 3744 publications), highlighting that this is probably the most studied protein in scientific world. This statistic reflects the fact that *P53* mutations are amongst the commonest molecular events in neoplasia: it is mutated in approximately 50% of human cancers and is possibly inactivated in all tumours through loss or upregulation of other genes in the *P53* pathway e.g. $P14^{ARF}$ and

MDM2 (Harris, 1996, Haupt *et al.,* 1997). *P53* is thought to be lost at a relatively late stage in colorectal cancer (Vogelstein and Kinzler, 1996). It is thought to be lost in up to 70% of colorectal cancers, marking the transition from benign colonic adenoma to adenocarcinoma (Vogelstein and Kinzler, 1996, Fazeli *et al.,* 1997). The precise reason for this loss at this stage is still unclear. However its role as a general mediator of the DNA damage response is thought to be important.

For many years it has been known that *P53* is a critical cellular mediator of the response to genotoxic damage *in vivo* and *in vitro* (Wyllie *et al.,* 1994). *P53* has been shown to induce apoptosis in response to a wide variety of insults and shown *in vitro* to also induce G1 and G2 cell cycle arrest via transcriptional activation of p21/WAF (Agarwal *et al.,* 1995). Recently, a series of significant advances have been made in the *in vitro* characterisation of the *P53* pathway. These include the identification of ever increasing types of cellular stress that upregulate *P53*; the identification of post-translational modifications in response to such stress; and the characterisation of proteins that interact with or are transactivated by *P53* (see figure 1.10)(for a recent review see Prives and Hall, 1999). One obvious goal of these studies is to link stimuli such as ionising radiation to a particular set of events which determine biological fate such as the engagement of apoptosis or cell cycle arrest. However, whether the system will be as straightforward as this remains to be seen. It has also been demonstrated *in vitro* that *P53* may have a direct role in maintaining genome integrity by preventing homeologous recombination, recognising and removing mismatches and participating in Nucleotide Excision Repair (Lee *et al.,* 1995, Janus *et al.,* 1999).

Figure 1.9: Postulated *P53* pathway, (adapted from Prives and Hall, 1999). Following a range of stresses, the P53 protein is modified. Thus far the proteins identified to modify P53 are mainly kinases: DNA-PK, ATM and ATR. Although as yet unproven, such modifications are postulated to render Mdm2 unable to bind P53 thus causing accumulation of P53. Depending on the specific cellular context, this could lead to apoptosis, cell cycle arrest or even senescence



One obvious reason for loss of *P53* in colorectal cancer is due to loss of the apoptotic response. A large number of studies have shown that basal levels of apoptosis in the murine intestine and adenomas are unaffected by *p53* status (Merrit *et al.,* 1994, Clarke *et al.,* 1994). However, post DNA damaging agents (e.g. gamma irradiation, 5-Flurouracil (5-FU), cisplatin) *p53* is necessary for the normal apoptotic response (Clarke *et al.,* 1994, 1997 Pritchard *et al.,* 1999). Thus chemotherapy treatment of a tumour may have selected for loss of *P53*, possibly explaining the association of *P53* loss with the later stage of tumourigenesis. However, if loss of *P53* is independent of treatment, then many tumours may have already lost *P53* prior to treatment. This raises clear doubts over the efficacy of treatments that are thought to work through *P53* dependent apoptosis. If they are seen to be effective this raises questions upon role of *P53* dependent apoptosis in chemoresistance.

Another potential reason for the loss of *P53* in colorectal tumourigenesis, is due to its role in governing apoptosis in response to hypoxia (Graeber *et al.,* 1996). This is of particular relevance to late tumour development, as regions of low oxygen and necrosis are common features of solid tumours. Loss of this apoptotic pathway has therefore been proposed as a key feature in tumour development and in the selection within tumours of more aggressive clones.

As yet, the relevance of DNA damage induced apoptosis to spontaneous malignancy is still unclear and difficult to investigate. Therefore assigning which out of p53 plethora of functions is crucial to tumourigenesis remains difficult.

1.4.5 The phenotype of the *p53*-deficient mice

Prior to the generation of mice deficient for *p53*, a number of different *in vitro* studies had suggested key roles for *p53* in initiating both apoptosis and cell cycle arrest. These predicted a severe phenotype for *p53* deficiency and it was therefore of some surprise that *p53* deficiency did not result in embryonic lethality as had

been reported for other cell cycle genes such as *Retinoblastoma (Rb)* (Harrison *et al.,* 1995). This led to the suggestion that *p53*'s true role was as a "guardian of the genome", with a primary function of protecting against genotoxic damage (Lane, 1992). Any role for *p53* in development was therefore initially discounted, however several lines of evidence have now made it clear that *p53* does indeed play a role in normal embryonic development. Data has been obtained in support of a role in embryogenesis from the careful analysis of *p53* breeding data. This indicated an apparent deficiency in the number of live born $p53^{-/-}$ female mice (Armstrong *et al.,* 1995, Sah *et al.,* 1995). When embryos were examined *in utero* and at the point of parturition, 23% of female null embryos were found to have neural tube defects, which presented as exencephaly from approximately day 12 of development, ultimately resulting in anencephaly at birth. Further support of the concept that *p53* might suppress germline mutations comes from the observation that a significant increase (60%) in exencephaly is seen in $p53^{-/-}$ female progeny derived from males irradiated prior to mating. Again this suggests that *p53* plays an important role in protection of the embryo against genotoxic stress. The reason why this manifests itself predominantly within females remains unclear, but this does reflect a similar sex bias seen in some human neural tube defects. These studies have demonstrated at least partial dependency upon *p53* for normal development. Our understanding of this role has now been extended by experiments using mice null for *Mdm2* which have shown that abnormal regulation of *p53* is also incompatible with normal development (Jones *et al.,* 1995, Luna *et al.,* 1995). On its own, *Mdm2* deficiency results in lethality, yet this is rescued in the absence of *p53*. The basis of this rescue is assumed to lie in the ability of Mdm2 to target *p53* for degradation. Thus, in the absence of functional *Mdm2*, *p53* is overexpressed resulting in lethality (see figure 1.10). Similar evidence for interaction between these two genes has been obtained using a transgenic approach to overexpress a *Mdm2* transgene and so cause loss of *p53* (Jones *et al.,* 1998).

Therefore although *p53* does appear to have a significant role in development, the fact that the majority of mice were viable allowed the testing of its role as a guardian of the genome *in vivo* (Lane, 1992)

1.4.5.1 Tumourigenesis in $p53^{-/-}$ mice

Three different strategies were employed to generate the first *p53* knockout mice in the early 1990's (Donehower *et al.,* 1992, Purdie *et al.,* 1994, Jacks *et al.,* 1994). All gave similar phenotypes with the majority of the $p53^{-/-}$ mice dying from lymphoma at around 6 months. This gave definitive proof that *p53* was a tumour suppressor gene, which previous clinical data and in vitro work had suggested (Harris and Holstein, 1993). However none of the $p53^{-/-}$ mice develop colorectal lesions.

Li-Fraumeni patients are germline heterozygotes for *P53*. Mice heterozygous for *p53* can be viewed as a relatively accurate model of the Li-Fraumeni Syndrome, as they develop tumours of a similar spectrum as those seen in Li-Fraumeni patients (Oestosarcomas, lymphomas and soft tissue sarcomas) (Malkin *et al.,* 1990, Srivastava *et al.,* 1990). However there are two notable exceptions to this, namely the high incidence of lymphoma in the mice and the absence of breast tumours (for recent reviews of mouse data see Loazona and Lui, (1999), Attardi and Jacks, (1999). *P53* heterozygotes develop symptoms of disease later than their *p53* null counterparts, with approximately 50% of mice succumbing to tumours by 18 months. As wild type mice live for up to 36 months, this age of death of the heterozygotes is often compared to Li-Fraumeni patients where affected individual have a 50% chance of developing cancer by the age of 30 (Donehower *et al.,* 1996). However, great care should be taken in making direct comparisons between the consequences of *p53* deficiency in man and mouse as many species specific differences exist, perhaps most notably in the strong predisposition to lymphoma observed in the mouse.

Within the mouse, strain dependent differences in phenotypes have readily been observed (Donehower *et al.,* 1995, Donehower *et al.,* 1996). For example, pure

bred 129SV $p53^{-/-}$ mice die from tumours much earlier than those crossed onto a C57Bl6 background. Furthermore, 50% of $p53^{-/-}$ males in 129SV develop testicular teratomas which are only observed rarely in other backgrounds. This latter observation probably reflects that p53 deficiency accentuates any natural tumour predisposition, as wild type 129SV mice show a spontaneous predisposition to testicular teratomas compared to other strains (VanMeyel *et al.*, 1998, Stevens and Little, 1954, Harvey *et al.*, 1995).

This strong predisposition for lymphoma in the mouse, may have masked a more subtle intestinal phenotype. To overcome this problem (as with the mismatch repair deficient mice), $p53^{-/-}$ mice was crossed to the $Apc^{Min/+}$ mice. The original studies suggested that there was no accelerated intestinal tumourigenesis in the ($Apc^{Min/+}$, $p53^{-/-}$) mice (Clarke *et al.*, 1995, Fazeli *et al.*, 1998). However there was an increase in pancreatic tumours and desmoid fibromas. Two caveats to this lack of this effect in the mouse was that $Apc^{Min/+}$ mice rarely develop carcinoma, probably due to their high burden of adenomas causing mortality. Thus if *p53* plays a role in the adenoma-carcinoma transition then this may not produce a phenotype in the mouse. Also (unlike the situation of ($Apc^{Min/+}$, $Msh2^{-/-}$) mice), ($Apc^{Min/+}$, $p53^{-/-}$) mice still die of lymphoma which could mean that a subtle intestinal phenotype could be missed. By crossing the ($Apc^{Min/+}$, $p53^{-/-}$) mice onto a uniform C57BL/6 background with known *Mom1* resistance alleles (resistance and sensitive), Hallberg *et al.*, (2000) showed that there was increased intestinal tumour multiplicity and invasiveness in ($Apc^{Min/+}$, $p53^{-/-}$) mice compared to the $Apc^{Min/+}$ mice at 90 days. This effect was quite subtle: on a resistance background the number of tumours increased from an average 5 to 13 whilst on the sensitive background they increased form 32 to 45. Again the increase in invasive phenotype was also relatively minor : 2/42 ($Apc^{Min/+}$, $p53^{-/-}$) tumours were invasive compared to 0/78 in $Apc^{Min/+}$ tumours and ($Apc^{Min/+}$, $p53^{+/-}$) tumours.
Therefore, *p53* plays a relatively small role in intestinal tumourigenesis in the mouse. Probably the best way to further analyse this phenomenon would be to use an intestinal specific *p53* knockout (e.g an intestinal specific cre mouse crossed to a *p53* floxed mouse).

p53 has been shown that to be involved in a myriad of functions, such that working out the significance of each of these is difficult. Recently the production of specific knockouts has help elucidate *p53* function. Probably the most crucial function of *p53* is its role as a transcription factor. Jiminez *et al.,* (2000) made a *p53* transactivation deficient mouse which showed defects in apoptosis and cell cycle arrest. Like $p53^{-/-}$ mice they also developed lymphomas and sarcomas. Thus it appears transactivation is a central component of *p53* tumour suppression. Choa *et al.,* (2000) also showed that *p53* transactivation deficient ES cells do not undergo apoptosis following UV damage. Targets of *p53* transactivation post genotoxic damage include *p21*, *Bax*, *Gadd45a*, *Mdm2*, *cyclin G1*, a large number of PIG genes (p53 Inducible Genes) and possibly *Msh2* (Bates and Vousden, 1996, Polyak *et al.,* 1997). Of these, *Bax* is a potent inducer of apoptosis, *p21* mediates cell cycle arrest and *Gadd45a* is important in protecting against genomic instability (Hollander *et al.,* 1999). Murine knockouts have been made of these genes. Interestingly there was no obvious phenotypes to the *Bax* and *p21* knockout mice (Deng *et al.,* 1995). In fact *p53* dependent apoptosis still occurred in the murine small intestine post DNA damage in the $Bax^{-/-}$ mice (Pritchard *et al.,* 1999b). Although there was no obvious phenotype in the mouse, $p21^{-/-}$ murine embryonic fibroblasts (MEFs) had a defect in the G1 arrest response (Deng *et al.,* 1995). Furthermore it has now been shown that $p21^{-/-}$ mice are in fact tumour prone although this only becomes apparent in mice over 16 months (Martin-Caballero *et al.,* 2001). $Gadd45a^{-/-}$ mice exhibit many similar symptoms to $p53^{-/-}$ mice. MEFs from $Gadd45a^{-/-}$ mice show genomic instability e.g aneuploidy, chromosome aberrations, gene and centrosome amplification. They also exhibit increased radiation carcinogenesis, exencephaly and an impaired G2 arrest to UV and alkylation damage (Hollander *et al.,* 1999,Wang *et al.,* 1999). However they do not show a deficiency in apoptosis. This similarity in phenotype to the $p53^{-/-}$ mice again highlights the importance of *p53* transactivation to its overall function. In summary, from these studies it appears that transactivaton domain of *p53* is crucial to *p53* function. As the transactivation domain is crucial to *p53*'s ability to induce apoptosis and arrest this argues that these phenomena are also important

for *p53's* role in tumour suppression. However despite being crucial for the induction of apoptosis after DNA damage, a role for p53 in tumour suppression in the murine intestine is still unclear showing that care should be taken in extrapolating a role for apoptosis to a role in tumourigenesis.

Through the interaction of *Msh2* and *p53*, this thesis will also examine other functions of *p53* that are possibly not dependent on its role as a transcription factor (Chapter 6).

1.4.5.2 *p53* and spontaneous mutation *in vivo*

The fact that the majority of $p53^{-/-}$ mice (and $Msh2^{-/-}$ mice) are viable has allowed questions to be posed addressing the importance of loss of gene function for phenotypically normal cells *in vivo*, when previously these questions had been restricted to (usually immortalised) cell lines *in vitro*. Although studies on such lines have produced invaluable data and will continue to do so, nearly all of these lines carry multiple genetic alterations. This makes the task of dissecting out the effect of individual genes very difficult.

The advent of knockout technology has not only produced a range of new *in vivo* systems, but it has also permitted the production of a new series of *in vitro* models. An obligatory step in the production of the knockout mice is the generation of targeted Embryonic Stem (ES) cells. If so desired, these can be rendered homozygous by either a second round of targeting or by the use of high levels of the appropriate antibiotic, which can select for conversion of the wild type allele. The availability of mutant cell lines are not however restricted to ES cells, as primary lines such as embryonic fibroblasts can relatively easily be derived from mutant strains. Such primary lines carry the considerable advantage that they will not have been selected for additional genetic change in culture, and so should parallel the genotype of the mutant strain

In each of these systems attempts have been made to determine the effect of genotype upon mutation rate. In reality these assays measure mutation frequencies, and broadly fit into two categories. First, those that that focus on a phenotypic change due to inactivation or overexpression of a somatic gene which is either autosomal or X-linked. These include changes resulting in loss of function at the *Dlb-1b* (Clarke *et al.,* 1997) and *HPRT* loci (Corbet *et al.,* 1999), overexpression of metallothinein (Jasani *et al.,* 1999) and ouabain resistance (Turker 1998). The second category includes those approaches which measure mutation frequency from exogenous transgenes, including *lacI* (Big Blue™, *lacZ* (Muta™) and *supF* (tRNA suppressor gene) (Turker, 1998).

The most commonly used *in vivo* assays in the mouse are the *Dlb1* assay, and assays using the *lacI* and *lacZ* transgenes (Winton *et al.,* 1988, Nishino *et al.,* 1995). The *HPRT* (Hypoxanthine phosphoribostltransferase) is the most commonly used assay for *in vitro* estimates (Corbet *et al.,* 1999). All of these techniques have drawbacks associated with them, however it is encouraging that many of the *in vivo* tests yield relatively similar frequencies (see Cosentino and Heddle, 1999).

In this thesis mutation frequency will be investigated at the *Dlb-1b* locus. The *Dlb-1* locus encodes for 2 lectin binding proteins. *Dlb-1a* is constitutively expressed on vascular endothelium. The *Dlb-1b* allele specifies binding of the Dolichos biflorus agglutinin to intestinal epithelium. Thus, in mice heterozygous for these alleles a single inactivating mutation at the *Dlb-1b* will abrogate the ability to bind this lectin in the intestinal epithelium. After cell proliferation and clonal expansion, mutations that occurred in the stem cell population will form clones that fail to bind a peroxidase conjugate of Dolichos biflorus agglutinin. These mutant clones can easily be visualised and scored to give an estimate of the mutation frequency.

As with the other approaches, the *Dlb-1* assay does suffer from some shortcomings. One drawback of this approach is that it is limited to the analysis of

intestinal mutation rates, as specific *Dlb1-1b* expression is restricted to the intestine and thus mutation frequency can only be estimated in this tissue. A further difficulty is that the locus has not yet been cloned and so questions relating to mutational spectra cannot be addressed (Buettner *et al.,* 1997). The assay does however possess a number of unique advantages over other approaches. Key amongst these is its ability to specifically score mutation within the stem cell population and to permit visualisation of the mutant clones *in situ* (Winton *et al.,* 1988). Furthermore, because the *Dlb-1* locus is an endogenous allele, there are few concerns over the i*n vivo* relevance of results, as have been raised following the use of an exogenous transgene.

When mutation frequencies are compared between the *Dlb1* assay and the transgenic mice Big Blue™ and MutA™, the transgenic models generally appear a little less sensitive (Cosentino and Heddle, 1999). You *et al.,* (1998) showed that one of the reasons for this was the presence of a large number of CpG sites in the *lacI* gene which are methylated *in vivo*. The *lacI* transgene usually consists of multiple concatemers (around 40) of the *lacI* gene within a lambda-like shuttle vector. This can be harvested from the mouse genome and packaged within the lamda phage. Bacteria are then infected on XGAL containing plates. After lysis those phages which contained a mutated copy of the *lacI* will produce clear plaques whilst those with a functional copy will produce blue plaques. The number of blue plaques therefore provides an estimate of mutation frequency (Nishino *et al.,* 1995, Buettner *et al.,* 1997, You *et al.,* 1998). Likewise the transgene used in the MutA ™ system is *lacZ,* which encodes the β-galactosidase gene. Here the numbers of mutants are counted by growing the phages on PGAL (Sigma) containing substrate, which prevents wild type *lacZ* plaques from growing (Gossen *et al.,* 1992).

One feature of the transgenic assays is that they allow the precise nature of the mutation to be determined. This can therefore be used to generate mutation spectra, the detail of which can often be used to rationalise apparently contradictory results. For example, one unexpected finding was that mutation

frequency in the *lacI* transgene did not increase significantly following treatment with X-rays, whilst a marked increase was observed at the *Dlb-1* locus (Tao and Heddle, 1994). The probable explanation for this is that X-Rays predominantly cause gross deletions, which may result in deletion of the entire shuttle vector. In such circumstances these events will be under-represented in the *lacI* assay but not the *Dlb-1* assay.

One potential difficulty with assays using endogenous loci is that there may be *in vivo* selection against mutants. For example, it has been shown that there is selection against HPRT deficient T lymphocytes cells in mice. Also, large deletions encompassing the HPRT locus can be selected against as a result of the deletion of flanking loci (Duebel *et al.,* 1996). Notably, these difficulties have not been reported for the *Dlb-1* assay. Some of these considerations may also hold true for the transgene based assays, as the site of transgene integration is thought to influence assay readout (Nishino *et al.,* 1996).

Despite the individual limitations of each approach, the assays described above have been shown to yield broadly consistent results, and to be capable of identifying increases in mutation following most types of DNA damage. These assays have now been applied to murine strains mutant for genes implicated in DNA repair. Such analyses have proven particularly powerful where use has been made of both the transgenic and endogenous gene-based assays. For example, an increase in mutation frequency *in vivo* in the *Msh2*$^{-/-}$ mouse has been reported using both the Big Blue™ mouse and *Dlb1* assay (Andrew *et al.,* 1998, Toft *et al.,* 1999). The Big Blue mouse showed an increase in all the three tissues studied: small intestine, thymus and heart. Comparison of the data generated by the two assays shows remarkable similarity: an approximate 10 fold increase was scored in the Big Blue mouse (3.1×10^{-5} in wild type intestine rising to 34×10^{-5} in the *Msh2*$^{-/-}$ intestine). A similar 7 fold increase was scored at the *Dlb-1b* locus (4 mutants per 10000 villi for wild type intestine rising to 28 mutants per 10000 villi in the *Msh2*$^{-/-}$ nulls intestine). Furthermore, both assays concurred in that heterozygosity for *Msh2* did not impact upon mutation frequency.

The proposed role for *p53* as a 'guardian of the genome' predicts that *p53* deficiency should lead to an increase in mutation frequency. Surprisingly, this has not been substantiated experimentally (Clarke *et al.,* 1997, Nishino *et al.,* 1995, Buettner *et al.,* 1997, Sands *et al.,* 1995). The initial report showed no increase in the spontaneous mutation frequency in the liver, spleen or brain of the Big Blue™ mouse when bred onto a $p53^{-/-}$ background (Nishino *et al.,* 1995). Subsequent sequencing of the entire lacI gene confirmed that there was no increase in either mutation frequency or spectra (Buettner *et al.,* 1997). One possibility was that *p53* deficiency only influenced the mutation rate in some tissue types. The most likely candidate tissue for such an effect was the thymus, as this is heavily predisposed to malignancy in the $p53^{-/-}$ background. However a similar study to those above extended the essentially negative finding to this tissue (Buettner *et al.,* 1996). When spontaneous mutation frequency in the small intestine was examined at the *Dlb-1b* locus, again this yielded no significant *p53*–dependent increase. Likewise when Corbet *et al.,* (1999), examined mutation frequency at the *HPRT* locus in ES cells, $p53^{-/-}$ ES cells again failed to show a significant elevation in mutation frequency.

Five potential explanations may be advanced to explain the observed lack of *p53*-dependency. First, these experiments were performed with 6-10 week old mice and it could be argued that insufficient time had elapsed for mutations to accumulate (Nishino *et al.,* 1995). Certainly this is possible, if *p53* is considered to be responding to DNA damage which will accumulate with age. However, since the majority of cells will have undergone many rounds of replication by 6-10 weeks of age, it seems unlikely that they will not have been exposed to substantial DNA damage (Nishino *et al.,* 1995). Second, the spectrum of mutations identified by these assays may be inappropriate for the type of instability produced by lack of *p53*. However, this possibility seems extremely unlikely given the concordance between the different types of assay.

A third possibility arises from the fact that a large proportion of *p53* mutations in human tumours are dominant negative, implying that the nullizygous state may be an inappropriate model for the study of *p53* as a tumour suppressor (Harris and Holstein, 1993). However, the vast majority of studies strongly argue that loss of function of *p53* is functionally equivalent, whether through the acquisition of a dominant negative mutation or through a homozygous null genotype. Although this difference is an important caveat to remember when considering the $p53^{-/-}$ mouse as a model for *p53* mediated tumourigenesis, it is extremely unlikely to bear any significance on the role played by *p53* in protecting against mutation in normal cells.

A fourth possibility is that *p53* is only relevant in guarding against increased mutation within abnormal or malignant cells. However, this also seems unlikely, as when Buettner *et al.,* (1996) analysed thymic lymphomas from *p53* null mice only 1 out of four tumours had an increased mutation frequency compared to normal thymus. In 4 tumours examined by Sands *et al.,* (1995), none were found to have an increased mutation frequency. This in itself is a fascinating observation, questioning the mutator hypothesis of cancer which attempts to explain why tumours acquire so many somatic mutations (far above the postulated mutation rate for somatic tissues) (see Loeb, 1998).

Finally, it may be that *p53* cannot be shown to play a role because it is essentially redundant. Recently, several *p53* homologues have been identified that could theoretically substitute for *p53* in the $p53^{-/-}$ mice. For example, the homologue *p73* has been implicated in *p53* independent apoptosis following both cisplatin treatment and ionising radiation (Gong *et al.,* 1999, Yuan *et al.,* 1999). Obviously, this cannot reflect complete redundancy, otherwise no exclusively *p53*-dependent phenomena (such as *p53*-dependent apoptosis) would be observed. However, given that mutation frequency is almost certainly determined by a number of interacting factors, redundancy remains a tenable explanation.

Given the current state of the field, it is not yet possible to discriminate between these possibilities. However, the fundamental observation that deficiency of *p53* fails to influence spontaneous mutation rate has challenged the view that *p53* plays a direct role that in DNA repair. It has also questioned the *in vivo* significance of *p53* dependent apoptosis and cell cycle arrest.

1.4.5.3 *P53* and apoptosis, clonogenic survival and mutation frequency in vivo

The preceding discussion has addressed the consequences of loss of gene function at spontaneous levels of DNA damage. These studies are clearly limited to the low levels of environmental insult that may exist in a normal laboratory animal house setting, and will therefore not address the consequences of exposure to defined types of DNA damage. Analysis using spontaneous levels of DNA damage also precludes the determination of a series of endpoints. Thus, apoptotic dependency can only be scored in circumstances that induce apoptosis. Similarly, clonogenic survival can only be scored when the majority of cells are lethally damaged. This is a particularly crucial endpoint to study as the emergence of malignant clones must be absolutely dependent upon the long term survival of the founder cell. For these reasons mice mutant for *p53* and *Msh2* have been studied following exposure to defined types of DNA damage.

Amongst the first studies performed using the *p53* knockout mice were those investigating the *p53*-dependency of apoptosis following genotoxic insult (normally ionising radiation). Initially Clarke *et al.,* (1993) and Lowe *et al.,* (1993) showed that unlike wild type thymocytes, which rapidly undergo apoptosis following treatment with ionising radiation and etoposide, $p53^{-/-}$ thymocytes were resistant. Heterozygotes were found to have an intermediate phenotype. However, $p53^{-/-}$ thymocytes were not resistant to the glucocorticoid methylprednisoline, nor to apoptosis induced following treatment with a calcium ionophore. These experiments showed that *p53*-dependence was restricted to certain types of stress, usually clastogenic damage. These observations gave rise to the notion that the

failure to engage apoptosis may be the critical predisposing factor to tumorigenesis in a *p53*$^{-/-}$ environment. Thus, cells exposed to DNA damage would not be deleted in the absence of *p53*, but would persist with a higher mutation burden and therefore a greater predisposition to malignancy.

Many different groups have attempted to directly test this hypothesis *in vivo*. As discussed above, *p53* deficiency apparently failed to influence the spontaneous mutation frequency. In order to analyse the effects of *p53* deficiency post DNA damage, Griffiths *et al.,* (1997) analysed short term primary cultures of IL7-dependent B cell precursors following exposure to X-rays. Here, they showed that *p53*$^{-/-}$ cells were resistant to apoptosis and that there was no gross difference in DNA repair as scored by the COMET assay. Clonogenic survival was measured and the *p53*$^{-/-}$ cells were found to have significantly enhanced survival. Mutation frequency was scored amongst the surviving cells, yielding an intriguing result. The frequency of mutants arising in the *p53*$^{-/-}$ cells was found to be equivalent to that previously scored for wild type cells. However, the total mutation burden (number of mutant clones) was considerably higher than predicted for wild type cells as a direct consequence of the difference in clonogenic survival. Taken together, these findings strongly argue for a *p53*-dependent increase in mutation burden occurring through increased clonality. These results are therefore essentially supportive of the central hypothesis that *p53* is the guardian of the genome (Wyllie *et al.,* 1994, Griffiths *et al.,* 1997).

The *in vivo p53*-dependency of apoptosis has also been investigated in the small intestine and in splenocytes. Analysis of both these tissue types again identified a wave of *p53*-dependent apoptosis peaking at 4-6 hours. However, a delayed *p53*-independent wave of cell death was also identified which was specific to the intestine and occurred at 48-72 hours (Clarke *et al.,* 1994, 1997, Merrit *et al.,* 1994,1997). These results highlight clear tissue specific differences in the dependence upon *p53* for the induction of apoptosis. When this *p53* independent wave was examined further, it was noted that the apoptotic bodies were much larger, suggesting that cells had entered apoptosis from a G2 block (Potten, 1990).

The precise significance of *p53*-independent death remains to be established, however it seems likely that it is effective in removing many of those cells survive by virtue of *p53* deficiency. This may well reflect *p73* activity, as has been reported in other systems (Gong *et al.,* 1999).

The tissue specific differences in *p53*-independent death may also explain two basic observations made in the $p53^{-/-}$ mice. First, the failure to see a *p53*-dependent difference in spontaneous mutation frequency in the intestine, as here there is an efficient alternative to *p53*-mediated death. Second, the strong predisposition to T-cell lymphoma in $p53^{-/-}$ mice, as this tissue relies exclusively on *p53* to mediate cell death.

1.4.5.4 Mutation and Clonogenic survival following DNA damage in the intestine

The small intestine provides an extremely well characterised system in which to study cellular proliferation, apoptosis and differentiation (for a review see Potten, 1997, Potten *et al.,* 1997). It is proposed that at the base of the crypt, there are approximately 4-6 stem cells. These are thought to be exquisitely sensitive to damage and undergo apoptosis under low levels of radiation (1Gy). However once these are destroyed other clonogenic cells can then substitute for the original stem cells. Following exposures of up to 9 Gy, there are thought to be an additional 6 clonogenic cells. Beyond this level of damage it is thought that up to 16-24 cells can then act as clonogenic cells (approximately a 1/3 of the crypt) (Roberts *et al.,* 1995).

The significance of the above is that clonogenicity can be investigated *in vivo* via an analysis of crypt survival (the micro-colony assay see Roberts and Potten 1994) and this data can subsequently be related to the ability to engage apoptosis. One problem with this assay is that relatively high levels of DNA damage must be used to deliver scorable crypt death. However, this assay circumvents one of the major

problems of *in vitro* assays in that it scores clonogenicity in a normal cellular setting.

Mutation frequency has also been investigated following exposure to DNA damage using those approaches discussed above. Again, the prediction from the central hypothesis is that *p53* deficiency will result in a significant increase in mutation frequency following treatment. Both clonogenicity and mutation frequency experiments have generated similar data. Hendry *et al.,* (1997) showed that there was essentially no increase in survival in $p53^{-/-}$ mice in the small intestine after gamma irradiation. In the large intestine, this study even indicated that $p53^{-/-}$ mice were more sensitive than wild type counterparts. Clarke *et al.,* (1997) examined mutation frequency at the *Dlb1-b* locus. At 200 and 400 rads, there was no significant difference between $p53^{-/-}$ and wild type mice. However a *p53*-dependent difference was observed at the higher dose of 600 rads. Notably, this increase was not seen to be *p53* gene-dose dependent, despite the fact that heterozygotes have an intermediate apoptotic phenotype.

One possible explanation for these results is that *p53*-independent apoptosis may be sufficient to remove cells harbouring DNA damage cells following exposure to low doses of ionising radiation, but that this mechanism is incapable of dealing with the damage inflicted at high doses. Such an explanation does not however rest easily with the failure to see a *p53*-dependent difference in clonogenic survival, as this predicts a difference at the high levels of DNA damage used in the reported assays.

In summary, the available data examining the relationship between apoptosis, clonogenic survival and mutation frequency i*n vivo* is at the least inconsistent with the simple hypothesis originally proposed. Indeed, the clearest published demonstration of *p53* dependency in intestinal epithelial cell survival appears to disassociate apoptosis and survival (Pritchard *et al.,* 1998). Here both low (40mg/kg) and high (400mg/kg) doses of the drug 5 FU were used. Both induced similar levels of apoptosis, which was *p53* dependent at the 24 hour peak

(although in the wild types the levels of apoptosis returned back to basal levels sooner in the animals treated at 40mg/kg). However it was only the mice treated at the higher dose which showed increased *p53*-dependent intestinal epithelial cell survival. Pritchard *et al.,* (1998) suggested that this was due to changes in cellular proliferation as mitotic cell indices and thymidine incoroparation fell to a much lower level in the mice treated at the higher dose. Thus, the presence or absence of a *p53* dependent growth arrest is suggested as the crucial factor governing clonogenic survival. These results again strongly suggest that simple correlations cannot readily be drawn between loss of apoptosis, increased clonogenic survival and mutation frequency.

This thesis will further investigate the significance of loss of apoptosis to long term clonogenic survival and mutation in both $p53^{-/-}$ and $Msh2^{-/-}$ mice (chapter 5).

1.4.6 DNA methylation and tumourigenesis.

Over the last few years, there has been a proliferation of publications noting that many genes are lost from tumours not through mutation but through hypermethylation of their promoters e.g *P16*, *MLH1*, *BRCA-1*, *P73*, *ATASE*, *E-Cadherin* (Baylin and Herman, 2000, Issa, 2000, Toyata *et al.,* 1999).

This methylation only occurs at cytosines which are 5' to a guanine (CpG dinucleotide). These CpG dinucleotides appear at about 10% of their expected frequency in the genome as 5-methyl cytosine can undergo spontaneous hydrolytic deamination to uracil/thymine. The majority of the remaining CpG dinucleotides are heavily methylated throughout the human genome (approximately 70%) (Bird, 1987,1992). However there are small streches of DNA called CpG islands of around 500-2000bp that are located around the transcriptional start site of around 40000 genes (approximately half of all genes). In virtually all cases these promoter CpG sites are unmethylated. It is these islands which have been shown to be hypermethylated in cancer.

As was mentioned earlier *MLH1* is often lost in sporadic colorectal and ovarian cancer through methylation (Herman *et al.,* 1998, Strathdee *et al.,* 1999). In these sporadic colorectal tumours, it was not just *MLH1* that was hypermethylated but a host of other promoters e.g *P16* and *ER* (oestrogen receptor) (Toyota *et al.,* 1999, Pao *et al.,* 2000). These were described as CIMP (CpG Island methylator phenotype) positive. Originally, Lengauer *et al.,* (1997b) proposed that these CIMP (+) lines were always MMR deficient, however this now seems not the case as both Toyota *et al.,* (1999) and Pao *et al.,* (2000) have shown CIMP positive, MMR proficient tumours and CIMP negative, MMR deficient tumours. However the majority of the CIMP+ lines do have *MLH1* hypermutation.

The precise reason for the CIMP phenotype is still unknown, however it leads to tumours that have inactivation of a specific subset of genes that have promoters susceptible to hypermethylation.

One of the most exciting potential therapeutic aspects of epigenetic changes like methylation is that they are potentially reversible (unlike mutation). As was described earlier, MLH1 was re-activated by DAC in human xenograft tumours so raising possibilities for chemotherapy (Plumb *et al.,* 2000). .

However it is not just hypermethylation that has been correlated to tumourigenesis, hypomethylation has also been shown to occur outside these CpG islands. Global hypomethylation has been shown to occur in many different tumours (Breivik and Gaudernack, 1999). However its role in tumourigenesis is less well understood. Experimental hypomethylation through inactivation of 5-cytosine DNA methyltransferase (DNMT1) (the enzyme which maintain methylation through cell divisions) has shown that cells with hypomethylation have an increased mutation rate and induce apoptosis via a *p53* dependent pathway (Chen *et al.,* 1998, Jackson-Grusby *et al.,* 2001). Interestingly enhanced genomic instability was characterised by increased mitotic recombination, chromosomal loss and duplication in the *Dnmnt1*$^{-/-}$ ES cells, all phenotypes of *p53*$^{-/-}$ cells as well. One hypothesis explaining the function of this methylation is

that it might repress transcription of repeated sequence such as Alu and transposons thus preventing genomic instability produced by transposition. Methylation may also cause chromatin changes to keep most of the genome late replicating thus possibly preventing rearrangement at replication (Baylin and Herman, 2000). However questions on the significance of global hypomethylation remain. Using the *Apc*$^{min/+}$ mouse model, it was shown that hypomethylation caused a reduction in the number of tumours in the *Apc*$^{Min/+}$ mouse. As the *Dnmt1*$^{-/-}$ mutation is lethal in mice, this was performed using (*Apc*$^{min/+}$, *Dnmt1*$^{+/-}$) mice injected with low levels of DAC (which causes loss of methylation). When the *Dnmt1*$^{+/-}$ mice were crossed to *Apc*$^{min/+}$ mice with the strongest *Mom* resistance allele almost no tumours were found (Cormier and Dove, 2000). Interestingly this was independent of *p53*. One possible explanation for this is that recently the *Apc* promoter has been shown to be hypermethylated in approximately 18% of colorectal cancers. Thus, as the AKR tumours show relative low levels of LOH at the *Apc*$^{+}$ locus compared to mice with other *Mom1* sensitive alleles, it is possible that here loss of *Apc* occurs through hypermethylation. This would then be suppressed in the *Dnmt1*$^{+/-}$ mice (Esteller *et al.,* 2000).

The importance of global hypomethylation to tumourigenesis is still unclear. However, specific hypomethylation does have a role in disease and tumourigenesis. The best example of this is in human cancer where 24% of mutations in p53 are G to A transitions at methylated CpG sites (Waters and Swan, 2000).

This thesis will investigate the impact that MBD4 (a methyl binding domain) protein (which is a mismatch specific thymine glycolase) has upon apoptosis, mutation and *Apc*$^{min/+}$ tumourigenesis. The MBD (methyl binding domain) proteins in more detail in chapter 8

In summary, this thesis aims to investigate the following principle questions:

First, what is the gene dependency of apoptosis in the small intestine to a range of DNA damage in respect to *Msh2, p53* and *Mbd4*?

Second, what is the significance of this gene dependent apoptosis in terms of long term survival and mutation accumulation?

Third, is the gene dependency of the apoptotic response retained in tumours?

Fourth, can therapies be tailored to the genetic background of the tumour?

2.0 Methods

2.1 Generation and Maintenance of Mice colonies

All mice were maintained under non-barrier conditions and given a standard diet (Harlan) (except those on aspirin diet) and water *ad libitum*.

The knockout mice used in this thesis are as follows: *Msh2*$^{-/-}$ mice (provided by Hien te Riele, see De Wind *et al.,* 1996), *p53*$^{-/-}$ mice (provided by C. Purdie see Purdie *et al.,* 1994), *Mlh1*$^{-/-}$ and *Pms2*$^{-/-}$ (provided by M. Liskay, see Prolla *et al.,* 1997), *Apc* $^{Min/+}$ (provided by W. Dove see Su *et al.,* 1992) and *Mbd4*$^{-/}$ mice (made by Jacky Guy from the Adrain Bird laboratory).

2.2 Genotyping of mice

2.2.1 DNA Extraction from tails

DNA was extracted from tails using the PUREGENE DNA EXTRACTION kit. Tails were lysed overnight in 500µl of cell lysis solution (Puregene) and 10µl of proteinase K (20mg/ml, Sigma), shaken at 37°C. Tails were left to cool at room temperature, 200µl of protein preciptation solution (Puregene) was added to each tube. These were vortexed and centrifuged at top speed for 5 minutes in a microfuge.

The supernatant was removed into a clean tube containing 500µl of isopropanol, vortexed and centrifuged at top speed for 5 minutes. The supernatant was poured and off and the DNA pellet was left to dry overnight. DNA was resuspended in 500µl DNA hydration solution (Puregene).

2.2.2. Genotyping of Mice via PCR

All genotyping of mice was done by genomic PCR (Polymerase Chain Reaction) from DNA extracted from tails (2.2.1). All PCR were done in 50µl volumes using 2µl of the tail DNA preparation.

2.2.2.1 *Msh2* PCR

The *Msh2* specific PCR reaction was designed by Toft *et al.,* (1999). The protocol for a 50µl reaction was as follows. The 50µl reaction mix contained 5µl PCR Buffer (Gibco), 2.5µl of 1% W1 detergent (Gibco), 1µl of each primer (10pmoles per µl) (OSWEL see below), 1µl of each DNTP (40mM) (Gibco), 2µl of $MgCl_2$ (50mM) (Gibco), 30µl of Autoclaved DDW (deionised distilled water), 1µl *Taq* polymerase (Gibco) and 2µl of template DNA (approximately 200ng). Reaction conditions were as follows: 94°C 5 minutes, then 30 cycles 94°C 1 minute (denaturation), 60°C 2 minutes (annealing) and 72°C 2 minutes (extension) and 10 minutes of 72°C. All PCR reactions were carried out using OmniGene Hybaid PCR machines.

18µl of PCR product was analysed on 4% TBE (Anachem) agarose gel stained with ethidium bromide (10mg/ml) (Sigma) and viewed under UV light. The *Msh2* knockout allele generated a 194bp allele whilst the wild type allele generated a 164bp allele.

Primer 1: CGGCCTTGAGCTAAGTCTATTATAAGG
Primer 2: GGTGGGATTAGATAATGCCTGCTCT
Primer 3: CCAAGATGACTGGTCGTACATAAG

2.2.2.2 P53 PCR

The p*53* specific PCR reaction was designed by Purdie *et al.,* (1994). The protocol for a 50µl reaction was as follows. The 50µl reaction mix contained 5µl PCR Buffer (Gibco), 2.5µl of 1% W1 detergent (Gibco), 1µl of each primer (10pmoles per µl) (OSWEL see below), 1µl of each DNTP (40mM) (Gibco), 2µl of $MgCl_2$ (50mM) (Gibco), 2 µl DMSO (Sigma), 28 µl of autoclaved DDW, 1µl *Taq* polymerase (Gibco) and 2µl of template DNA (approximately 200ng). Reaction conditions were as follows: 94°C 5 minutes, then 30 cycles 94°C 1 minute (denaturation), 62°C 1 minute (annealing) and 72°C 1 minute (extension) and 10 minutes of 72°C. All PCR reactions were carried upon OmniGene Hybaid PCR machines.

18µl of PCR product was analysed on 2% TBE agarose gel. The *p53* knockout allele generated a 600bp band of whilst the wild type allele generated a 700bp allele.
primer 1 GTGGTGGTACCTTATGAGCC
primer 2 CATCGCCTTCTATCGCCTTC
primer 3 CAAAGAGCGTTGGGCATGTG

2.2.2.3 *Apc*$^{Min/+}$ Genotyping

Apc$^{Min/+}$ genotyping was determined by digestion of PCR product by HindIII (Gibco) as described Luonga *et al.,* (1994). The protocol for a 50µl reaction was as follows. The 50µl reaction mix contained 5µl PCR Buffer (Gibco), 1µl of each primer (10pmoles per µl) (OSWEL see below), 1µl of each DNTP (40mM) (Gibco), 2.5 µl of $MgCl_2$ (50mM) (Gibco), 33.5 µl of autoclaved DDW, 1µl *Taq* polymerase (Gibco) and 2µl of template DNA (approximately 200ng). Reaction conditions were as follows: 94°C 5 minutes, then 30 cycles 94°C 1 minute (denaturation), 60°C 1 minute (annealing) and 72°C 1 minute (extension) and 10 minutes of 72°C. All PCR reaction were carried on OmniGene Hybaid PCR machines.
16 µl of product was digested by HindIII (Gibco) for 2 hours at 37°C and products analysed on a 4% TBE agarose gel. The digested wild type fragment generated a 111bp band whilst the digested *Min* fragment produced a 123bp band.

Primer 1: TCTCGTTCTGAGAAAGACAGAAGCT
Primer 2: 5' TGATACTTCTTCCAAAGCTTTGGCTAT

2.2.2.4. *Mlh1* PCR

The *Mlh1* specific PCR reaction was designed by Edelmann *et al.,* (1995). The protocol for a 50µl reaction was as follows. The 50µl reaction mix contained 5µl PCR Buffer (Gibco), 2.5µl of 1% W1 detergent (Gibco), 1µl of each primer (10pmoles per µl) (OSWEL see below), 1µl of each DNTP (40mM) (Gibco), 2µl of $MgCl_2$ (50mM) (Gibco), 30µl of autoclaved DDW, 1µl *Taq* polymerase (Gibco) and 2µl of template DNA (approximately 200ng). Reaction conditions were as follows: 94°C 5 minutes, then 30 cycles 94°C 1 minute (denaturation), 60°C 1 minute (annealing) and 72°C 1

minute (extension) and 10 minutes of 72°C. All PCR reactions were carried out using OmniGene Hybaid PCR machines.
Products were analysed on a 2.5% TBE gel. The wild allele generated a 258bp band and the knockout allele generated a 198bp band.

Primer 1:AGGAGCTGATGCTGAGGC
Primer 2:GATCTCGACGGTATCGATAAGC
Primer 3:TTTCATCTTGTCACCCGATG

2.2.2.5. Pms2 PCR

The Pms2 specific PCR reactions was designed by Baker *et al.,* (1995). As there is competition between the primers 2 separate reactions are done: 1 for the knockout allele and one for the wild type allele. Both protocols use the same reaction mix. The protocol for a 50µl reaction was as follows. The 50µl reaction mix contained 5µl PCR Buffer (Gibco), 2.5µl of 1% W1 detergent (Gibco), 1µl of each primer (10pmoles per µl) (OSWEL see below), 1µl of each DNTP (40mM) (Gibco), 2µl of $MgCl_2$ (50mM) (Gibco), 30µl of autoclaved DDW, 1µl *Taq* polymerase (Gibco) and 2µl of template DNA (approximately 200ng). Reaction conditions for the wild type allele was as follows: 94°C 5 minutes, then 30 cycles 94°C 1 minute (denaturation), 48°C 1 minute (annealing) and 72°C 1 minute (extension) and 10 minutes of 72°C. Reaction conditions for the targeted allele was as follows: 94°C 5 minutes, then 30 cycles 94°C 1 minute (denaturation), 60°C 1 minute (annealing) and 72°C 1 minute (extension) and 10 minutes of 72°C. All PCR reactions were carried out using a OmniGene Hybaid PCR machines.
Products were analysed on a 2.5% TBE gel.
Primer 1 (used in both reactions): TTCGGTGACAGATTTGTAAATG
Primer 2 (Mutant): TTTACGGAGCCCTGGC
Primer 3 (Wild type): TCACCATAAAAATAGTTTCCCG

2.2.2.6. *Mbd4* PCR

The *Mbd4* specific PCR reaction was designed by Jacky Guy (unpublished). The protocol for a 50µl reaction was as follows. The 50µl reaction mix contained 5µl PCR

Buffer (Gibco), 1µl of each primer (10pmoles per µl) (OSWEL see below), 1µl of each DNTP (40mM) (Gibco), 2.5µl of $MgCl_2$ (50mM) (Gibco), 30µl of autoclaved DDW, 1µl *Taq* polymerase (Gibco) and 2µl of template DNA (approximately 200ng). Reaction conditions were as follows: 94°C 5 minutes, then 30 cycles 94°C 1 minute (denaturation), 65°C 1 minute (annealing) and 72°C 1 minute (extension) and 10 minutes of 72°C. All PCR reactions were performed on a OmniGene Hybaid PCR machines.

Products were analysed on a 2.5% TBE gel. The wild allele generates a 322bp band and the knockout allele generates a 469p band.

Primers

1. AAGGTGGCACCTAGAGCTCTGTCG
2. GGATATTCGGTGCTGTCGCTCG
3. GTCGGTTTATGCAGCAACGAGACG (Mutant)
4. CAAACTGGCAGATGCACGGTTACG (Mutant)

2.3 Apoptosis- inducing agents, quantification of apoptosis, clonogenic survival and mutation frequency.

2.3.1 Reagents and administration

For apoptosis studies 8-12 week old mice were given intraperitoneal (i.p.) injections of either Temozolomide (Gift from Malcolm Stevens), N-Methyl-Nitrosourea (NMNU) (sigma isopac), MNNG (sigma), Nitrogen Mustard (Sigma), Cisplatin (Faulding Pharmaceutical/ David Bull), or 5-FU (5-Flourouracil) (Faulding Pharmaceutical/ David Bull).

Temozolomide was prepared fresh and first dissolved in DMSO (10% v/v) and made to a final concentration with Phosphate Buffer Saline (PBS) (Gibco). MNNG was prepared fresh and first dissolved in DMSO (10% v/v) and made to a final concentration with corn oil (sigma). NMNU was bought as an isopac and dissolved to appropriate concentration with PBS with 0.05% Glacial Acetic Acid. Nitrogen Mustard was dissolved to the appropiate concentration with DDW. Both Cisplatin and 5FU were bought as solution (10mg/ml).

Mice were exposed to γ-irradiation using a ^{137}Cs source at 0.27Gy min^{-1} for 15 min, so that each animal received a dose of 5,10 and 15 Gy.

2.3.2 Quantification of apoptosis

At each indicated time point following injection, a minimum of three animals were killed and the small intestine removed, flushed with water and fixed overnight in methacarn (4 parts methanol, 2 parts chloroform, 1 part acetic acid). Samples were wound into 'swiss rolls' and stored in 70% ethanol prior to staining.

Haematoxylin and Eosin (H&E) stained sections were made and apoptosis scored through the use of the Highly Optimised Microscopic Environment (HOME) microscope (Clarke *et al.*, 1994).

Apoptosis was identified through its morphological appearance. Apoptosis is recognisable in the intestine through the appearance of smooth membrane bound apoptotic bodies. Cell shrink to produce a halo around the apoptotic bodies with clear chromatin condensation within the nuclei. The nuclei also stains a much redder colour (see Kerr *et al.,* 1972, Wyllie *et al.,* 1980).

A separate individual assigned each slide a reference number and thus data scored blind. Running Means values were generated prior to counting which showed that 50 half crypts produced a robust running mean when scoring apoptosis in normal intestine. When apoptosis was scored in lesions, all cells were scored in type I and type II lesions and a random field of 500 cells in adenomas (Kongkantuutn *et al.,* 1999). Running mean values were generated for each lesion type which showed that 10 single crypts lesion, 10 complex lesions and 4 adenomas produced robust running means.

To address the possibility of intra-observer error sample slides were scored on 3 separate occasions. These counts did not significantly differ from each other (the degree of error was always below 10%). To check for inter-observer error, slides were counted by an independent observer. This process was facilitated by the HOME microscope as individual assessment of apoptosis could be checked by another observer.

All experiments were initiated at the same time of day (10 hours am) in order to control for the effects of diurnal changes in susceptibility to apoptosis since a clear circadian rhythm has been shown to exist for the induction of apoptosis in the murine small intestine. A minimum of fifty half crypts were scored per animal.

2.3.3 Assessment of Clonogenic Survival: Micro-colony assay of clonogenic survival.

The micro-colony assay was performed as described by Potten, (1990), Hendry *et al.,* (1997). Briefly, 72 hours after injection with cytotoxic agents, murine small intestines were removed, and divided into three sections: top, middle and bottom. Each section was cut into small pieces and bound into a bundle with 3M surgical tape.

These were fixed in 10% formalin and embedded. Histological cross sections were made and the numbers of surviving crypts were then counted around the circumference of the intestines. As all mice were harvested at the same time and comparison between genotypes are being made, there was no rationale for applying any correction factor (see Hendry and Potten ,1985). Crypts were designated as alive if they had more than 5 consecutive live epithelial cells in the crypt (see Hendry *et al.,*. 1997). Again running means were generated and 10 circumferences per slide produced a robust running mean of numbers of live crypts. Doses of 10 – 20mg/kg were used for cisplatin and 1-10mg/kg of Nitrogen Mustard according to Ijiri and Potten (1983,1987).

2.3.4 Mutation frequency at the *Dlb-1b* locus

The *Dlb-1* assay is a specific locus assay in which somatic mutations are detected that inactivate the polymorphic genetic locus *Dlb-1b* which encodes a lectin binding site (see Winton *et al.,* 1988). The assay is autosomal with the *Dlb-1* locus located on the mouse chromosome 11. The *Dlb1-1b* allele specifies the binding of the Dolichos *biflorus agglutinin* to intestinal epithelium. The *Dlb1-1a* allele specifies the binding of the Dolichos *biflorus agglutinin* to vascular epithelium. In mice *Dlb1-b/Dlb1-a* any inactivating mutation of the *Dlb1-b* gene leads to loss of the ability to bind the lectin in the intestine. After cell proliferation and clonal expansion, mutations that occurred in the stem cell population of the intestine result in the formation of a clone that does not bind a peroxidase conjugate of Dolichos *biflorus agglutinin* (EY Laboratories). These unstained clones are easy to score in the wholemounts of the small intestine.

For this assay, experimental cohorts were derived by crossing mutant mice (in this thesis the mutation experiment were performed in mice singly mutant for either *Msh2*$^{-/-}$, *Mlh1*$^{-/-}$, *Pms2*$^{-/-}$ or *Mbd4*$^{-/-}$) to two different C57Bl/6 strains, one of which was homozygous for the *Dlb-1a* allele and one of which was homozygous for the *Dlb-1*b allele. Mice were subsequently intercrossed from these two lines to generate mice heterozygous at the *Dlb-1* locus and segregating for all possible mutant genotype.

2.3.5 Wholemount Method for scoring mutation frequency at the *Dlb1-b* locus.

For spontaneous mutation frequencies mice were taken at the appropriate age (as noted in text). This was done at a range of ages for wild type, *Msh2*$^{+/-}$ mice, *Msh2*$^{-/-}$ mice, *Mlh1*$^{-/-}$ mice, *Pms2*$^{-/-}$ mice and *Mbd4*$^{-/-}$ mice.

For assessing induced mutation frequency following DNA damage, 8-12 week old mice were exposed to cytotoxic agent 3 weeks prior to harvesting. This gives time for clonal expansion as described above.

The method was as follows. After killing the mouse, the small intestine was removed and flushed with ice cold PBS. The second fifth of the intestine was removed and one end tied off. This was flushed with 5% formalin/PBS (Sigma) to expand intestine. The tied end was cut off and the intestine was again flushed with ice cold PBS.

The piece of intestine was pinned down at one end with pins (Watson and Doncaster) and the mesentry removed. The other end of the intestine was then stretched and pinned. The intestine was then cut open and pinned flat.

Intestines were fixed for one hour in 10% formalin (Sigma). They were washed with PBS and incubated with demucifying solution (see below) for 30 minutes .The plate was the flooded with PBS and mucus pipetted off.

The plate was then incubated in 0.5% BSA (Bovine Serum Albulmin) (Sigma) / PBS for 10 mins. The intestines were then incubated in the *Dlb1* peroxidaes conjugate (EY Laboratories) overnight in 1:200 concentration in 0.5% BSA/PBS.

The plates were washed and developed using Diaminobenzidine (DAB) (Sigma). Two 20mg tablet of DAB was dissolved in 40mls of DAB buffer (see below). 80µl of 1% H_2O_2 was then added and the DAB solution poured onto the guts for 5 minutes. After development, plates were stored in 70% ethanol until they are scored. Mutation frequency was scored per 10000 villi. This was done through scoring the number of non-staining villi in 50 fields of 200 villi.

Demucifying solution : 1:1:2:6 (Glycerol: 0.1M Tris: 100% ethanol: Saline)

For 100ml:

10 ml Glycerol (Sigma)

10ml 0.1M Tris (pH 8.2) (Fisher)

20ml 100% Ethanol (Fisher)

60ml Saline (Fisher)

340mg Dithiothreitol (DTT) (Sigma)

DAB Buffer: 100 ml pH 7.6

24ml 0.2M Tris

38ml 0.1M HCl (Fisher)

38ml DDW

0.0681mg Imidazole (Sigma)

2.4 Monitoring of cohorts

2.4.1 *Msh2* $^{-/-}$ and p53$^{-/-}$ mice

Mice were checked daily for symptoms of disease. The predominant cause of death in *Msh2*$^{-/-}$, *p53*$^{-/-}$ and multiply mutant mice in these two genes is lymphoma. Mice with thymic lymphomas have a hunched, puffing appearance whilst mice with lymphoma of the spleen (splenomegally) have a swollen, hunched appearance. Other tumours in these mice are sarcomas and akanthomas which are can be seen on the mice. When mice were ill, they were culled and dissected. Organs and tumours were removed into 10% formalin for histology and gut 'roll' performed as above.

For microsatellite analysis the tumour and a piece of tail were removed and snap frozen in liquid Nitrogen and stored at -80˚C. DNA was then extracted from them as above and Microsatellite analysis performed by Lucy Curtis.

For EMSA (Electrophoreic Mobility Shift Assay) and Western analysis for p53 functionality (see below) mice were irradiated with 5GY of irradiation 6 hours prior to culling.

2.4.2 $Apc^{Min/+}$ and ($Apc^{Min-/+}$ $Mbd4^{-/-}$) cohort

Mice were monitored daily for signs of disease. $Apc^{Min/+}$ and ($Apc^{Min/+}$, $Mbd4^{-/-}$) mice develop intestinal neoplasia (Su *et al.,* 1992). The timing of intestinal neoplasia in $Apc^{Min/+}$ mice has been shown be highly dependent of the *Mom1* locus (Modifier of Min) (Cormier *et al.,* 2001). Thus all death cohorts were performed on a congenic background for Min, the C57BL6 Mom allele. The symptoms of intestinal neoplasa in the $Apc^{Min/+}$ mice is rapid weight loss, anaemia, blood in faeces and a hunched appearance. $Apc^{Min/+}$ mice also rarely developed mammary akanthoma.

After culling, tissues were removed, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 10μM and stained with haematoxylin and eosin prior to microscopic analysis. Scoring of intestinal lesion was achieved by removing the entire intestine at necropsy and flushing with Phosphate Buffered Saline and mounting *en face*. These preparations were then fixed in methacarn (4:2:1 methanol, chloroform and glacial acetic acid). Lesions were then scored macroscopically. Intestine were then wound into a "swiss" roll which were subsequently embedded in paraffin and then sectioned as above

2.4.3. Aspirin Cohort.

2.4.3.1 Administration of dietary aspirin at weaning

C57BL/6 mice wild type and heterozygous for the Apc^{Min} mutation were placed on diet containing either 0, 200, or 400mg/kg of aspirin (Harlan/Tekad). These levels of exposure are comparable with the highest doses used in previous studies (Mahmoud *et al.,* 1998, Barnes and Lee, 1998, Williamson *et al.,* 1999, Chiu *et al.,* 2000) Mice were monitored everyday for signs of disease: generally manifesting itself as anaemia, loss of weight and a hunched appearance.

Two experiments were performed: one where mice were killed at 150 days of age and one where mice were killed where they showed signs of disease (see 2.4.2).

2.4.3.2 Permanent administration of aspirin

Matings segregating for progeny which were $Apc^{Min/+}$, $Msh2^{-/-}$ or ($Apc^{Min/+}$, $Msh2^{-/-}$) were placed on aspirin diet containing either 0 or 400mg/kg of aspirin prior to conception and throughout pregnancy and lactation. Progeny were weaned onto appropriate diets. Mice were killed when they showed signs of disease, generally intestinal neoplasia or lymphoma (see 2.4.1 and 2.4.2).

2.5 Primary Culture

2.5.1 Primary Murine Embryonic Fibroblast (MEFS).

$Msh2^{-/-}$, ($Msh2^{-/-}$, $p53^{+/-}$) and ($Msh2^{-/-}$, $p53^{-/-}$) MEFS were generated from crosses of ($Msh2^{-/-}$, $p53^{+/-}$) mice. Upon mating male mice produce a sperm plug in the female vagina which can easily be seen by eye. Primary MEFs were made from day 13-14 embryos, with day ½ being the day when the plug was first observed.

On day 13 ½ the female was culled and the embryos removed. The embryos were killed via cutting off of the head. They were dipped into 70% ethanol to make them sterile and then chopped up using a sterile scapel. At this point a small piece of the embryo was frozen for making DNA for genotyping (see 2.2).

After the embryo was finely chopped, 5ml of 2.5 % trypsin in PBS (Gibco) was added and the embryo was left for 5 minutes at 37°C. After trypsination, 10 ml of fibroblast media (see below) was added and cells were centrifuged at 1000 rpm for 5 mins. The supernatant was then removed and the pellet was resuspended in 10ml of media and plated on $10cm^2$ tissue culture dishes (NUNC). MEFs are grown at 37°C , 5% CO_2. Cells were confluent within 2 days.

Fibroblast Media:
500ml BHK21 Glasgow MEM media (Gibco)
50ml Foetal Calf Serum (Gibco)
5 ml Penicillin/ Streptomycin (100X Gibco)

2.5.2 MEF Clones.

From confluent plates of day 2 MEFs, fibroblasts were plated to grow clones for MSI and p53 functionality analysis. Unlike ES cells which produce rounded discrete clones (see Corbet *et al.,* 1999), fibroblast clones can be rather dispersed. Therefore a range of different cloning densities were plated. Confluent plates were trypsinised with 5ml of 1 x Trypsin/EDTA (Gibco) which was neutralised with 10ml of media. Centrifugation of cells at this stage is surprisingly incompatible with the formation of clones. Thus from the 15ml typsin and media mix 1µl, 5µl, 10µl, 20µl,50µl, 100µl and 1ml were plated on 10cm^2 tissue culture plates 10ml of media. Cells were re-fed the next day and then left for 2 weeks.

For immunohistochemistical analysis of p53 in clones, MEFs were grown on chamber slides.

For Western analysis, clones were pooled from plates and protein extracted (see 2.6.2).

For MSI analysis, clones were visualised by staining with Giesma (sigma). Clones were then picked and DNA made from them as in 2.5.1.

2.6 Protein analysis

2.6.1. Immunohistochemistry

2.6.1.1 p53 immunohistochemistry from fibroblast clones

Unlike formalin or methacarn fixed mouse tissues, no antigen retrieval steps are required for the p53 immunohistochemistry on fibroblasts clones. To induce p53 in the clones, cells were irradiated with 50J/M of UV-C and left for 6 hours. The cells were then washed with PBS to remove any traces of serum and fixed in pre-cooled (in the freezer) acetone/methanol for 5 minutes. The slides were then frozen at -70˚C until usage.

After thawing at rooming temperature, slides were washed with Tris Buffered Saline (sigma) with 0.1% Tween 20 (Sigma) (TBST). To block endogenous peroxidases, cell were blocked in 0.5% H_2O_2 (Sigma) in Methanol (Fisher Scientific) for 15 minutes. Cells were then washed in running tap water, then 5 lots of 2 minutes washes in TBST were performed.

To block any non-specific antibody binding, cells were blocked with 20% Normal Swine Serum (Dako) in TBST for 15 minutes. Cells were then blocked in Avidin D and Biotin (Dako) for 15 minutes. After washing 3 times in TBST, cells were incubated for 2 hours with the primary CM5 p53 antibody (Novacastra) at a concentration of 1 in 250 at room temperature.

Cells were washed 3 times with TBST and incubated in secondary antibody: biotinylated Swine anti-rabbit (Dako) at a concentration of 1:400.

After washing 5 times with TBST, cells were incubated in the Avidin biotin complex (ABComplex)/ HorseRadish peroxidase (HRP) (Vector laboratories) for 30 minutes and developed using DAB after a further 5 washes in TBST.

Slides were washed in running water and counterstained with haemoxytoxin (2 minutes). They were mounted in aquamount (Sigma) and sealed with nail varnish.

2.6.1.2 BrdU immunohistochemistry.

Mice were injected with 0.25ml of BrdU (bromodeoxyuridine) (Amersham) for 2 hours prior to harvesting.

The staining was done on paraffin embedded, methacarn fixed intestines. 3μM sections of intestines were cut onto Poly-L-lysine slides (Sigma). Sectioned were dewaxed by placing into xylene for 20 minutes. They were rehydrated through graded ethanol solutions (absolute alcohol, 74 % ethanol and 64% ethanol).

After a quick wash in water, slides were shaken at 60°C for 10 minutes in 1M HCl for antigen retrieval. They were washed in PBS and then blocked for 20 minutes in 1.5%

H_2O_2. Slides were incubated in 1% BSA/PBS for 20 minutes and then incubated in BrdU conjugate (Roche) at a concentration of 1in100. Slides were washed in PBS and then developed in DAB.

They were then stained with haemotoxylin for 2 minutes. Slides were then dehydrated in graded ethanol solutions (64%, 74%, absolute alcohol) and cleared in xylene. They were then mounted using DPX mounting medium (Sigma).

2.6.1.3 β-Catenin immunohistochemistry

Slides were cut and rehydrated as in 2.6.1.2. Antigen Retrieval was performed by microwaving slides 3 times for 15 minutes in Dako Antigen Retrieval solution. Slides were washed in TBS and blocked in the same way as 2.6.1.2. Cells were incubated in normal rabbit serum (Dako) for 20 minutes.

Slides were incubated in primary β-Catenin antibody (C19220, Signal Tranduction labs) for 1 hour at room temperature at a concentration of 1 in 50. After 3 washes in TBST, slides were incubated for 30 minutes with a rabbit anti-mouse biotynalated secondary (Dako) at a concentration of 1 in 200. Slides were washed and incubated in the ABComplex-HRP for 30 minutes. Slides were then developed with DAB, rehydrated and mounted as in 2.6.1.2.

2.6.1.4. Cyclin D1 immunohistochemistry

Slides were heated to 60˚C for 3 hours. They were then rehydrated as in 2.6.1.2. and placed into high pH Antigen Retrieval Solution (Dako) at 99˚C for three hours. Slides were cooled to room temperature for 20 minutes and then rinsed in TBS. Immunohistochemisitry was then performed according to the catalogue of the DAKO Envision Plus System kit (Dako cat K4006). Briefly, slides were blocked in peroxidase block for 5 minutes and washed with TBS. Slides were incubated with primary Cyclin D1 antibody (DCS-6, Novacastra) overnight at 4˚C at a concentration of 1 in 100. Slides were washed in TBS and the HRP secondary (Dako kit) was added for 30 minutes at a concentration of 1 in 400. Slides were the washed, developed with DAB and mounted as described in 2.6.1.2.

2.6.2 Immunoblotting of proteins

2.6.2.1 Protein extraction

2.6.2.1.1 Extraction from pooled fibroblast Clones

Fibroblasts were washed with ice cold PBS and then scraped off into 5ml of PBS and transferred to a 20ml universal. The flask was then washed with a further 5 ml of PBS, which was also transferred to the universal. Cells were centrifuged at 1000 rpm (revs per minute) for 5 minutes and supernatant removed.

Cell pellets were resuspended in 200µl of RIPA buffer (see below) and vortexed until the pellet had dispersed.

Cells were then sheared through a green needle (21G) and transferred to an eppendorf and centrifuged at 10000 rpm at 4˚C.

The supernatant was then removed, aliquoted and snap frozen into liquid Nitrogen and stored at -70˚C until use.

2.6.2.1.2 Extraction from murine tissues.

Tissues were removed from sick animals and snap frozen at -70˚C until extraction. A small piece of frozen tumour was then crushed using a mortar and pellet in liquid Nitrogen until it was a fine powder.

Using liquid nitrogen, this powder was then poured into a universal and the liquid nitrogen was left to evaporate off.

400µl of RIPA buffer was then added and tissue was sheared, centrifuged and aliquoted as described in 2.6.2.1.1.

RIPA buffer:

50mM Tris HCl pH7.5 (Fisher)

150mM NaCl (Fisher)

1% Nonidet p40 (Sigma)

0.5% sodium deoxycholate (Sigma)

0.1% SDS (Fisher)

To 10ml of RIPA buffer, 1 mini-cocktail tablet (Roche) of protease inhibitors was added

2.6.2.2 Determination of protein concentration

For estimation of protein concentration a microscale variant of Bradford's dye of binding method was using used (Bradford 1976). 5-10µl of protein sample was added to 1ml of Bradford reagent (BioRad), mixed and allowed to stand for 10 minutes before measurement of A_{595}. In each case absorbance measurements were performed against blanks containing an equal volume of the Bradford reagent.

These absorbance measurements were then correlated with freshly generated calibration curve (0-25µg of bovine serum albumin in 1ml of bovine serum albumin in 1ml Bradford Reagent) to estimate the protein concentration of the unknown sample.

2.6.2.3 Western Analysis

For Western analysis, proteins are run on denaturing polyacrylamide gels (10% gels for p53 westerns and 15% gels for p21 Westerns).

Protein samples were equalised with RIPA buffer so that all samples were 20µg and of equal volume (20µl). They were then boiled for 5 minutes in 4 x Loading Buffer containing β-mercaptoethanol, quenched on ice, centrifuged and loaded onto the gel. Gels were run for 2 hours at 125V in running buffer or until the protein markers (Gibco) had separated.

Gels were then blotted on PDVF membrane (Millipore) in transfer buffer overnight at 15 mA. Prior to transfer PDVF membrane was soaked in methanol (Fisher) for 30 minutes.

After transfer, blots were blocked in TBS/0.1% Tween/10% Marvel (TTM) for one hour. Blots were incubated with primary antibodies were incubated for one hour at room temperature. The p53 antibody (rabbit polyclonal) CM5 (novacastra) was used at a concentration of 1 in 200 whilst the p21 antibody (goat polyclonal) SC-672G (Santa Cruz) was used at a concentration of 1 in 100. Blots were washed 3 times in TTM and incubated in secondary antibody (Santa Cruz): anti-rabbit-HRP (p53) and anti-goat-HRP (p21) at a concentration of 1 in 2000.

Blots were then washed in TBS and visualised using ECL plus (Amersham) on ECL film (Amersham). To confirm equal loading after blotting, blots were then stained with Ponso Red (Sigma).

Reagents

15% Loading Gel	10% loading Gel	10% Stacking Gel
3.33ml DDW	6.65ml DDW	3.57ml DDW
11.69ml 30% acrylamide (1:29)	8.35ml 30% acrylamide (1:29)	1.70ml 30% acrylamide (1:29)
9.37ml 1M Tris HCL pH 8.8	9.37ml 1M Tris HCL pH 8.8	0.62ml 1M Tris HCL pH6.8
250µl of 10% SDS (Fisher)	250µl of 10% SDS	50µl of 10% SDS
72µl of 25% APS (Fisher)	72µl of 25% APS	33µl of 25% APS
13.2µl of Temed (Sigma)	13.2µl of Temed	3.6µl of Temed

4x Loading Buffer

200mM Tris HCl pH 6.8

400mm Dithiothreitol (DTT)

8% SDS

0.4% Bromophenol blue

40% Glycerol

10x Running Buffer: For 1 L

30.2g Tris

188g Glycine (Fisher)

Transfer Buffer For 1L:

800ml DDW

200µl methanol

2.9g Tris

14.5g Glycine

2.6.3. Electrophoretic Mobility Shift Assay (EMSA)

2.6.3.1 Protein extraction

EMSA were used to test whether the DNA binding activity of p53 was still intact. Thus unlike Western analysis where a whole cell extract was prepared, a nuclear extract is required for EMSAs. This was done using a high salt concentration in the Nuclear Extraction buffer.

For cell extractions, cells were scraped from plates in cell lysis buffer (2.5×10^7 nuclei per ml). Cells were pelleted via centrifugation at 2000 rpm at 4°C and resuspended in nuclear extraction buffer (2.5×10^7 nuclei per ml). The nuclei were then rocked for 1 hours at 4°C and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was then removed and frozen in liquid nitrogen and kept at -70°C until use.

For protein extractions from animal tissues, tissues were crushed into a fine powder as in 2.6.2.1.2. and protein extracted as above except that 400µl of cell lysis solution and nuclear extraction buffer was used. Protein concentrations were determined as in 2.6.2.2.

Cell Lysis Buffer:
20mM HEPES pH7.6 (Sigma)
20% glycerol
10mM Nacl,
1.5mM MgCl2 (Fisher)
0.2mM EDTA (Fisher)
0.1% Triton x-100 (Sigma)
1mM DTT (Sigma)
10µg/ml pepstatin (Roche)
10µg/ml leupeptin (Roche)
15µg/ml aproteinin (Roche)

Nuclear Extraction Buffer

The same as cell lysis buffer apart from 500mM NaCl.

2.6.3.2 EMSA protocol

p53 consensus and mutant oligonucleotide (from Santa Cruz) were end labelled with γ-ATP (ICN) using T4 polynucleotide kinase (Gibco). They were filtered using a Biogel p-4 gel (Biorad).

10μl of nuclear extract (25μg of protein from p53 wild type cells/tissues or 50μg of protein from p53 heterozygous cells/tissues) was mixed with an equal volume of binding buffer containing 0.1-0.3ng of 32P labelled probe. These were incubated for 30 minutes at room temperature. For antibody supershift analysis 2μl of anti-p53 antibody was added (AB-1 Oncogene Science/ Santa Cruz) for an additional 20 minutes before electrophoresis. 0.05% bromophenol blue/xylene blue (sigma) was added to each sample and loaded onto a 5% non-denaturing acrylamide gel (made up in TBE, Sigma). The gel was ran at 150V in TBE for 2-3 hours.

Gels were dried on a vacuum dryer (Biorad) and exposed overnight to BIOMAX MS-1 film in an autoradiography cassette with intensifying screens at -70˚C

Binding buffer

50mM NaCl
40mM HEPES (Sigma)
3mM MgCl2 (Sigma)
10μg/ml pepstatin (Roche)
10μg/ml leupeptin (Roche)
15mg/ml aproteinin (Roche)
1μg of Salmon sperm DNA (Stratagene)

Chapter 3: O^6-Alkylguanine-DNA-alkyltransferase (ATase) does not affect apoptosis following alkylating agents in the murine small intestine

3.0 Introduction

A central focus of this thesis is the gene dependency of apoptosis following alkylating agents. The murine small intestine offers an ideal experimental system to allow the study of both apoptosis and mutation frequency in response to DNA damaging agents such as DNA alkylating agents (Clarke *et al.*, 1997; Merritt *et al.*, 1997 Potten *et al.*, 1997).

The DNA repair protein O^6-alkylguanine-DNA-alkyltransferase (ATase) is expressed in the murine small intestine and is known to be up-regulated in a *p53*-dependent manner in response to DNA strand breaks following ionising irradiation (Rafferty *et al.,* 1996). ATase functions by recognising and removing specific alkyl lesions from DNA (Pegg & Byers, 1992). Substrates include O^6-methylguanine which is believed to be the major pre-mutagenic lesion induced by alkylating agents. ATase mediated repair occurs via the transfer of the alkyl group to a cysteine residue in the ATase protein, a process which is stoichiometric and autoinactivating (Pegg, 1990). ATase therefore confers protection against the mutagenic effects of alkylating agents, many of which are of clinical importance as anti-tumour agents such as Temozolomide and Dacarbazine (von Hofe & Kennedy, 1992; Dumenco *et al.,* 1993; Liu *et al.,* 1996b; Sanderson & Shield, 1996). Failure of ATase to repair O^6-methylguanine results in O^6-methylguanine:thymine mispairs following DNA replication and it is postulated that these mediate cell death *via* mismatch repair (Karran & Bignami, 1994). The DNA mismatch repair enzyme *MSH2* is known to bind to and recognise O^6-methylguanine:thymine mispairs and is also essential for a large proportion of apoptosis observed in the murine small intestine following exposure to alkylating agents (Duckett *et al.*, 1996; Toft, *et al.* 1999). In fact, in many tumours ATase activity is often lost through promoter hypermethylation (Wheeler *et al.,* 2000)

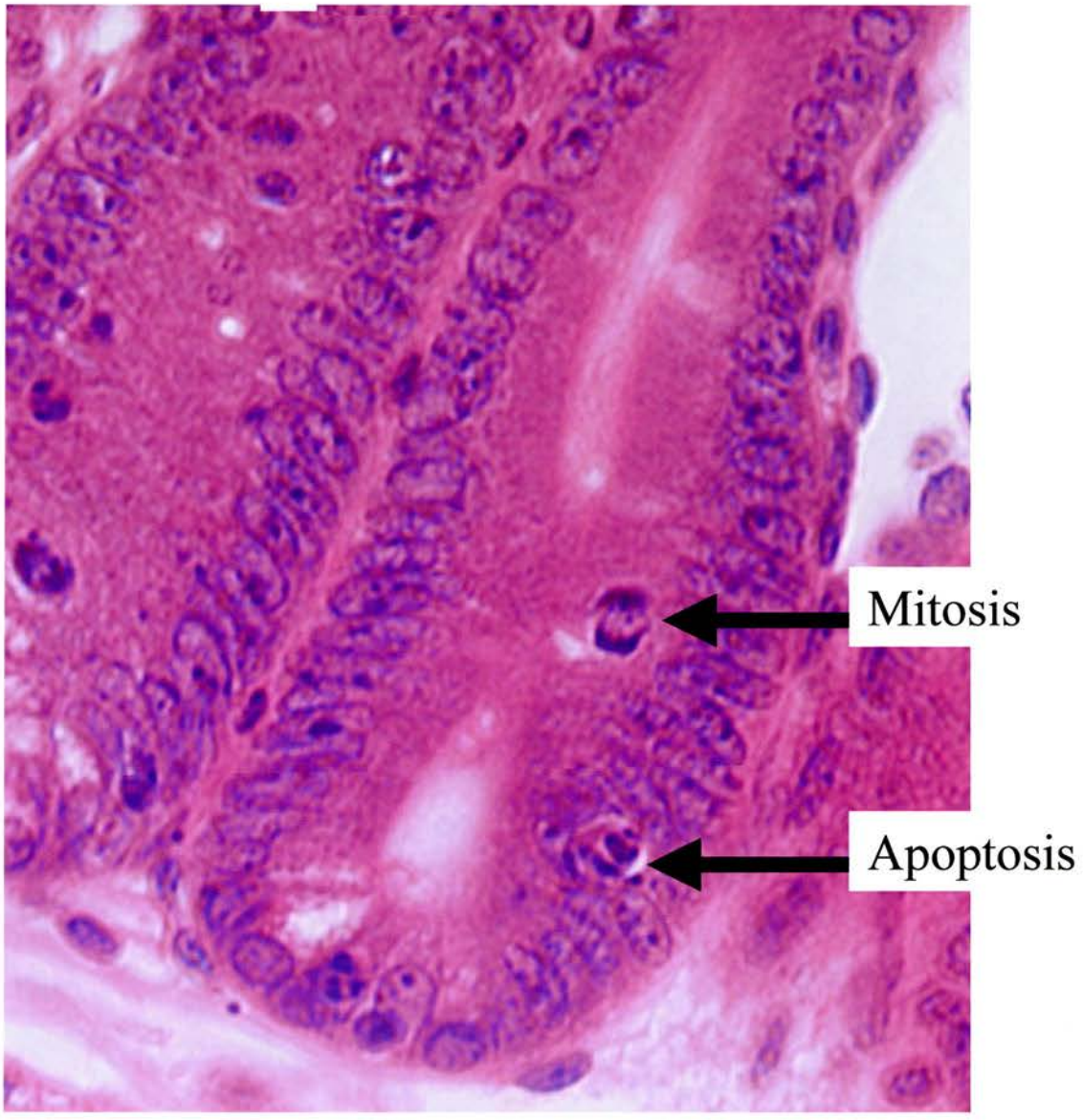
Therefore prior to characterising the importance of mismatch repair proteins in mediating the apoptotic response following alkylation damage, the significance of ATase to apoptosis in the murine small intestine was investigated. This was done

by comparing the induction of apoptosis in untreated wild type mice and in mice that had ATase chemically inactivated through administration of BeG (O^6-Benzylguanine).

BeG is a competitive and irreversible inhibitor of ATase which acts by binding to the -CH_3 cysteine acceptor site of ATase forming *S*-Benzylcysteine (Dolan *et al.,* 1990: Pegg *et al.,* 1993). BeG bound ATase is inactive and is subsequently degraded. Cellular ATase activity can be restored only by *de novo* protein synthesis (Pegg & Byers, 1992) and its depletion by BeG has been proposed as a useful adjuvant to methylating and chloroethylating treatment of tumours in the clinic (Wedge & Newlands, 1996). Adjuvant therapy of BeG combined with an alkylating agent offers the potential to use lower, and hence less toxic doses of chemotherapeutic drug, and may also be of benefit in overcoming tumour resistance associated with an up-regulation of ATase (Dolan *et al.,* 1993).

Administration of 60mg/kg BeG functionally depletes ATase activity in the small intestine for a period between 1 and 12 hours post-drug administration (Toft *et al.,* 1999, 2000)

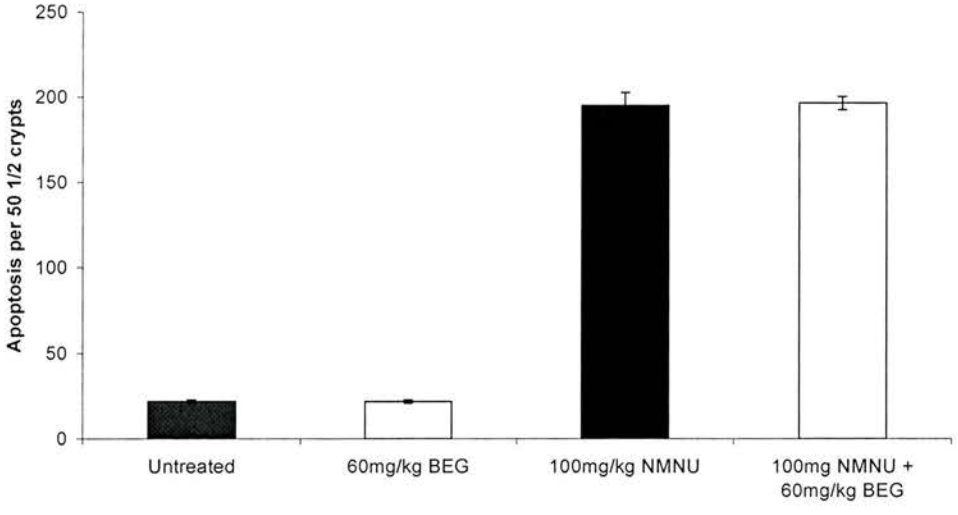
Figure 3.1 Small intestinal crypt showing cells undergoing apoptosis and mitosis. The cell underoing apoptosis shows chromatin condensation, nuclear membrane blebbing and a halo. Most apoptosis occurs in the stem cell region of the crypt (position 4-6) whilst mitosis generally occurs at positions higher up the crypt (as shown here) (Potten, 1997).



3.1.1 Apoptosis in the murine small intestine following exposure to alkylating agents is not affected by BeG treatment.

The peak induction of apoptosis in the murine small intestine following alkylating agents is approximately 6 hours (for timecourse see Toft *et al.,* 1999). Therefore apoptosis was scored in 50 half crypts 6 hours post 50mg/kg NMNU. Two cohorts of mice were used, either untreated or injected with 60mg/kg BeG two hours prior to injection with NMNU. To ensure that BeG treatment did not induce apoptosis, mice were injected with BeG alone and compared to mock treated mice.

Figure 3.2 BeG does not affect apoptosis per se or following NMNU.
Apoptosis was scored per 50 ½ crypts in intestinal enterocytes. Addition of 60mg/kg BeG did not alter either basal levels of apoptosis or apoptosis induced following 100mg/kg NMNU.



Following exposure to BeG alone, levels of apoptosis were not elevated above background levels. This conclusion is further consolidated by Toft *et al.,* (1999,2000) who showed that apoptosis was not affected over 72 hour timecourse following BeG treatment. There was also no difference between levels of apoptosis induced by NMNU and NMNU + BeG (Mann Whitney, P=0.38).

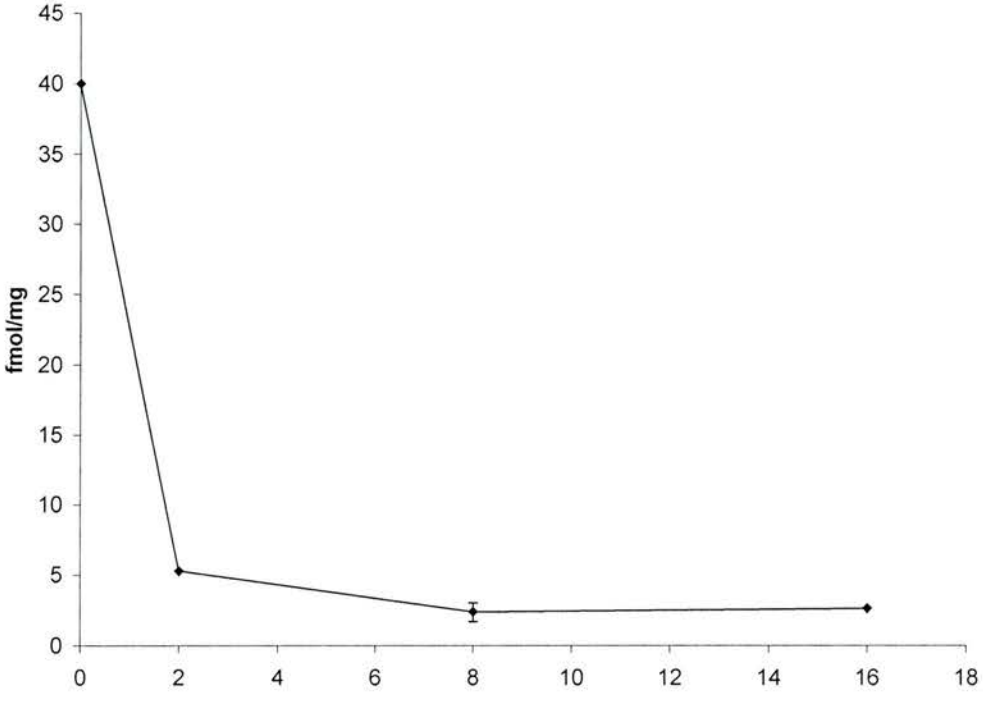
Therefore BeG treatment does not appear to affect levels of apoptosis induced by the alkylating agent NMNU.

3.1.2 Low levels of alkylating agents inactivate ATase in the murine small intestine

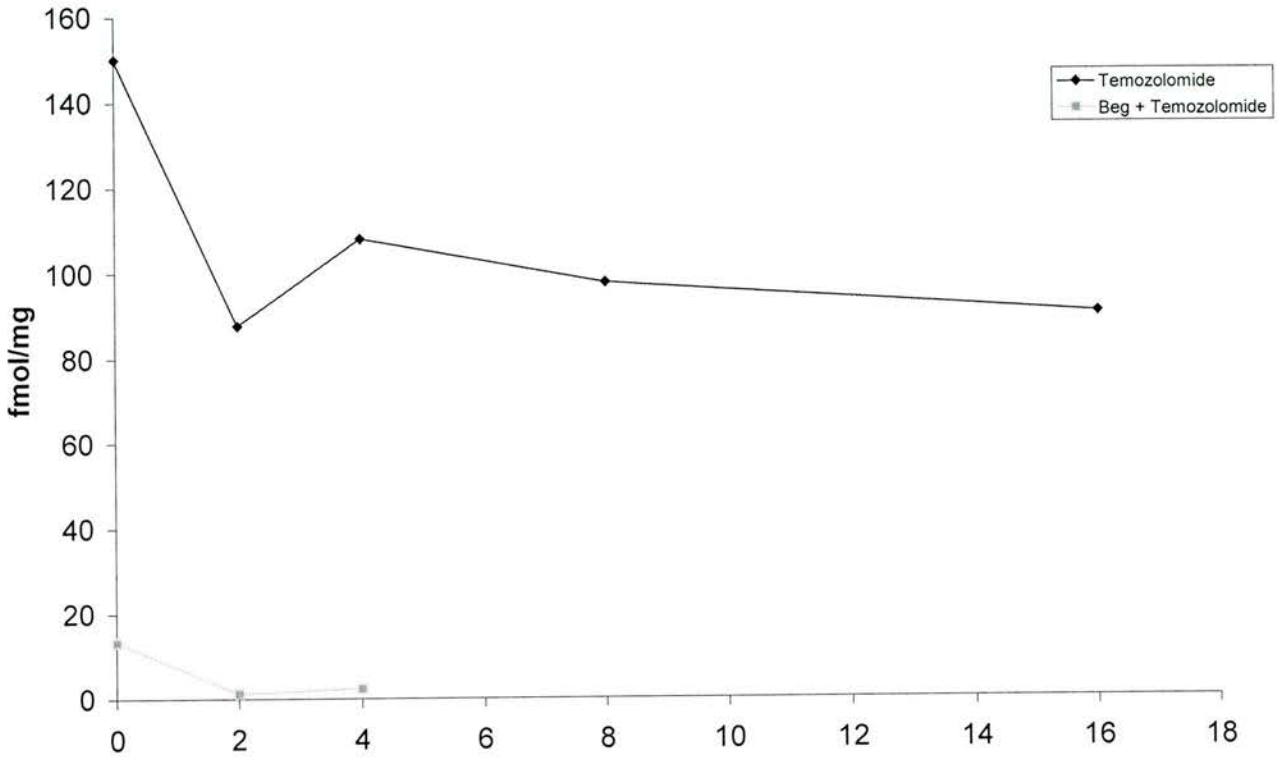
Toft *et al.,* (1999, 2000) had previously shown that BeG treatment also did not affect apoptosis following temozolomide treatment (from 2 to 100mg/kg). One possible explanation for the failure to observe a difference in apoptosis following BeG treatment is that injection of alkylating agent alone is sufficient to inactivate ATase by depleting cellular pools of active ATase and therefore rendering BeG treatment irrelevant (even at very low doses). To investigate this further (in collaboration with the Geoffrey Margison laboratory) levels of Atase was measured in temozolomide and mock treated mice, 6 hours post exposure. Samples of small intestine and liver (for a comparision) were harvested and snap frozen in liquid nitrogen. ATase activity was assayed from these tissues by Michelle Wood.

Figure 3.3 The effect of temozolomide on ATase activity. ATase activity was measured in fmol/mg protein in the liver (A) and in the small intestine (B) following a single injection of temozolomide. Error bars represent SD.

A) Small intestine



B) Liver



As can be seen, the resting levels of ATase in the murine small intestine (approximately 40fmol/mg) were much lower than that in the liver (150fmol/mg). Importantly even low levels 2-16mg/kg of temozolomide are enough to inactivate ATase in the small intestine, with no activity being found at 100mg/kg. The liver however showed only a small decrease in levels of ATase from 2-16mg/kg though at 100mg/kg a large reduction in ATase activity (10fmol/mg +/-6) is evident.

3.2 Discussion

No induction of apoptosis was associated with BeG treatment. This excluded the possibility that either BeG itself or depletion of ATase could induce apoptosis in the murine small intestine. In the absence of detectable ATase activity, exposure to NMNU induced apoptosis. The levels of apoptosis were similar to those observed in BeG untreated controls. Thus, BeG treatment neither reduced nor augmented the prevalence of apoptosis in the intestine following alkylation damage.

Such failure to modify phenotype may have occurred because *in vivo* exposure to methylating agents ablates cellular ATase activity by depleting pools of unmethylated ATase, so rendering BeG treatment irrelevant (Pegg & Byers, 1992; Lacal *et al.,*1996). To address this possibility, temozolomide was administered at decreasing doses in the presence and absence of BeG. Similar levels of apoptosis were scored in both BeG treated and untreated mice, suggesting that either ablation of ATase was occurring following exposure to very low levels of temozolomide, or that BeG mediated inactivation of ATase is irrelevant to the induction of apoptosis following methylation damage.

Ablation of the ATase activity might be predicted to lead to an increase in mutation frequency scored in this manner. Toft *et al.,* (2000) examined mutation frequency at the *Dlb-1b* locus following increasing doses of NMNU in both the presence and absence of BeG. However, pre-treatment of mice with BeG failed to elevate the mutation rate following NMNU treatment. As with the effect of BeG treatment upon the induction of apoptosis, the observed failure to modify the mutation rate in the murine small intestine may be interpreted in two ways. First, ATase activity may not be relevant to protection against mutation in this tissue. Second, exposure to the methylating agent alone may be sufficient to inactivate ATase activity irrespective of BeG activity. Although it is not possible to absolutely differentiate between these two possibilities, it is clear that BeG pretreatment does not alter either the apoptotic response or mutation frequency.

There is increasing interest in the possible use of BeG clinically as an adjuvant to alkylating agent chemotherapy to overcome tumour resistance mediated by ATase and also to potentiate the cytotoxic effects of the chemotherapy (Wedge & Newlands, 1996; Kurpad *et al.,* 1997). For a number of reasons the potential clinical benefits of BeG may be overestimated. First, alkylating agents themselves are capable of inactivating ATase activity by depleting cellular pools (Pegg & Byers, 1992). Second, the addition of BeG to cells treated with MNNG or Temozolomide is known to increase mutation rates and chromosome aberrations within cells, although there is a proportional increase in apoptosis (Lukash *et al.,* 1991; Bean *et al.,* 1994; Chinnasamy *et al.,* 1997). Third, from the data presented in this thesis, it is clear that in the murine small intestine ATase depletion does not elevate *in vivo* apoptotic levels following methylating DNA damage. Furthermore, Toft *et al.,* (2000) showed that BeG can alter the metabolism of drugs or carcinogens, e.g. Dacarbazine, and may therefore be a cause of unexpected adverse drug reactions if used clinically.

In conclusion, these results in conjuction with Toft *et al.,* (2000) have shown that BeG treatment does not affect basal levels of apoptosis *per se* or apoptosis induced by the alkylating agents temozolomide, MNNG or NMNU in the murine small intestine. Toft *et al.,* (1999) also showed that there are no differences in the levels of ATase between *Msh2* and wild type mice. Taken together with the very low levels of ATase in the small intestine which are rapidly inactivated, this argues that ATase levels will not affect experiments in the murine small intestine. Therefore any differences observed will instead reflect true gene dependency in the knockout mice

Chapter 4: The role of Mlh1 and Pms2 in apoptosis induction following the alkylating agents: NMNU and Temozolomide

4.0 Introduction

This chapter investigates the importance of the MutL homologues *Mlh1* and *Pms2* in the induction of the normal apoptotic response following exposure to the alkylating agents temozolomide and NMNU. Previously it has been shown that *Msh2* can bind the O6meG lesion and loss of *Msh2* causes a reduced apoptotic response following temozolomide and MNNG *in vivo* (Toft *et al.,* 1999). Andrew *et al.,* (1998) and Toft *et al., (*1999) showed that this ability to engage apoptosis is an important indicator for mutation as *Msh2*$^{-/-}$ mice injected with NMNU (N-methyl-nitrosourea) and temozolomide have much higher levels of mutation than wild type mice. Taken together, these studies suggested *in vivo* that *Msh2* deficiency may predispose to malignancy not only through failed repair of mismatch DNA lesions, but also through the failure to engage apoptosis.

The replicative cycling model (shown in figure 1.8.) argues that functional MMR is required to signal apoptosis following alkylation damage (Karran and Bignami, 1991). Although the precise role of the MutL homologues *Pms2* and *Mlh1* have not been completely characterised, they are both essential for MMR. *MLH1* and *PMS2* have been shown to be mutated in HNPCC, though *MLH1* is mutated much more frequently (*MLH1* is lost in approximately 60% of HNPCC whilst *PMS2* is lost in less than 1 % of families (Vogelstein and Kinzler, 1996). Therefore if the model of replicative cycling is correct the prediction is that the apoptotic response following the alkylating agents: temozolomide and NMNU should be reduced to the same levels as those observed in the *Msh2*$^{-/-}$ mice.

Previous *in vitro* studies have suggested that this should be the case. Unike wild type immortalised MEFS, *Mlh1*$^{-/-}$ MEFS do not undergo a G2 arrest in response to NMNU exposure (Buermeyer *et al.,* 1999b). A similar failure to undergo a G2 arrest is observed in immortalised *Mlh1*$^{-/-}$ following cisplatin, 5-FU, 6-TG and gamma irradiation (Fink *et al.,* 1997b, Davis *et al.,* 1998, Meyers *et al.,* 2001). In each of these experiments no differences in the levels of apoptosis were observed, though this is thought to represent that *in vitro* these agents preferentially induce

arrest rather than apoptosis (at the doses used in these studies). Thus far the only *in vitro* study highlighting a role for *Mlh1* in apoptosis rather than arrest was following H2O2 treatment which elicited *Mlh1*-dependent apoptosis (Hardman *et al.,* 2001)

Immortalised *Pms2*$^{-/-}$ MEFS have been shown to have a compromised apoptotic response to ionising radiation (albeit a at very high dose of gamma irradiation) (Zeng *et al.,* 2000). *Pms2*$^{-/-}$ mice have also been shown to have significantly higher levels of mutation in reporter transgenes in skin following gamma irradiation than wild type mice (Xu *et al.,* 2001). However whether this was due to failed apoptosis cannot be ascertained as there was no investigation of levels of apoptosis in the skin.

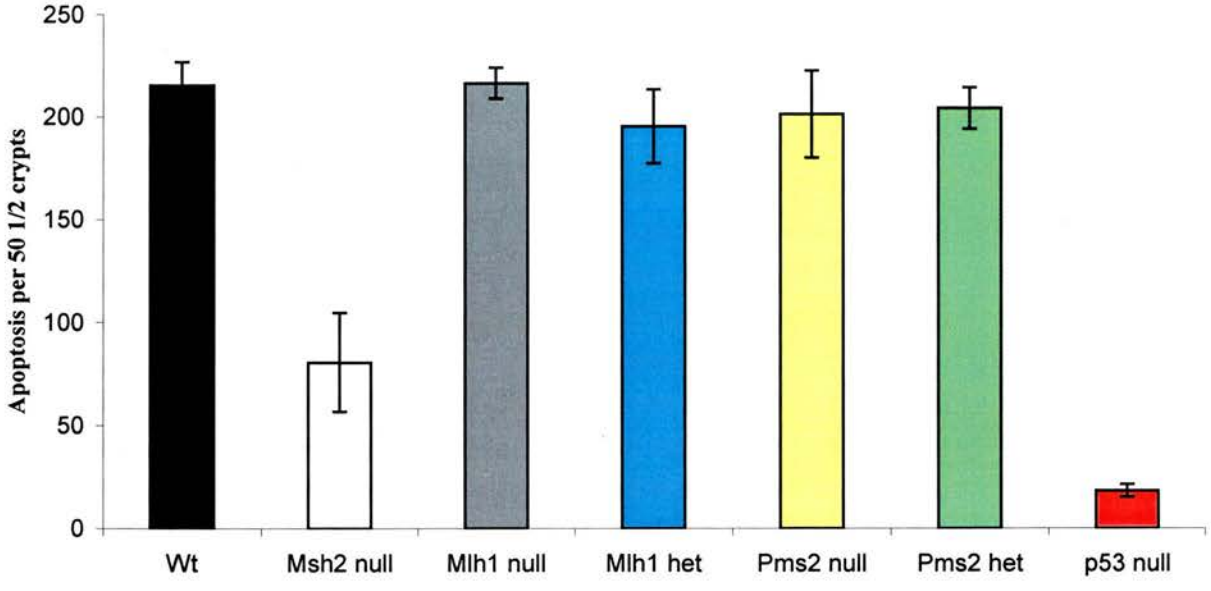
Fishel's (1998,1999) model of direct signalling again proposes that functional MMR is required for signalling apoptosis. However unlike replicative cycling (which is a more passive process), Fishel argues that the MMR can sense very high lesional loads and thus signal death instead of repair. As yet all the experiments on the molecular switch hypothesis has focussed on the MSH homologues, thus it still theoretically possible that this direct signalling could occur in the absence of the MLH homologues.

4.1. Results

4.1.1 Normal apoptotic response following 50mg/kg NMNU treatment

In order to investigate whether *Mlh1* and *Pms2* were important for induction of apoptosis following alkylation damage, the apoptotic response following 50mg/kg NMNU was investigated. This was the same dose that Andrew *et al.,* (1998) used to show that *Msh2* was important for suppressing mutation following NMNU treatment.

Figure 4.1 Apoptosis following NMNU treatment. Apoptosis was scored 6 hours following treatment with NMNU. Black bar, wild type mice; Open bar, *Msh2*$^{-/-}$ mice; Grey bar, *Mlh1*$^{-/-}$ mice; blue bar, *Mlh1*$^{+/-}$ mice; Yellow bar, *Pms2*$^{-/-}$ mice, Green bar, *Pms2*$^{+/-}$ mice and pink bar, *p53*$^{-/-}$ mice. At least three mice were used for each timepoint and error bars represent SD.

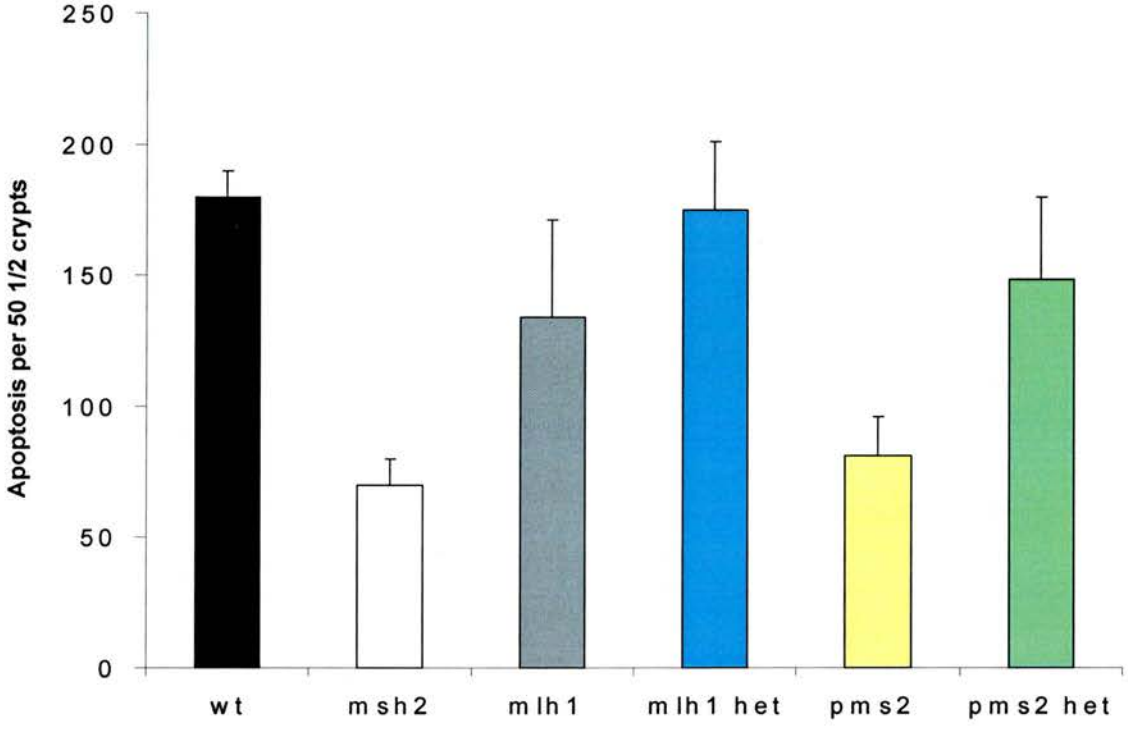


Apoptosis was scored six hours following treatment with 50mg/kg NMNU. As expected there was a reduced level of apoptosis in the *Msh2*$^{-/-}$ mice (p=0.04, Mann Whitney) and an abrogated response in the *p53*$^{-/-}$ mice (p=0.04, Mann Whitney) compared to wild types. This is consistent with the gene dependency of apoptosis after the alkylating agents seen in *Msh2*$^{-/-}$ and *p53*$^{-/-}$ mice following MNNG and temozolomide treatment. However surprisingly both *Mlh1*$^{-/-}$ and *Pms2*$^{-/-}$ mice had a normal apoptotic response to NMNU compared to wild type mice (Mann Whitney, *Mlh1*$^{-/-}$ mice (n=5) compared to wild type mice (n=5), p=0.77, Pms2 compared (n=4) to wild type mice, p=0.18). No gene dose dependency was observed in either *Mlh1*$^{+/-}$ and *Pms2*$^{+/-}$ mice.

4.1.2 Reduced apoptotic response following 100mg/kg temozolomide

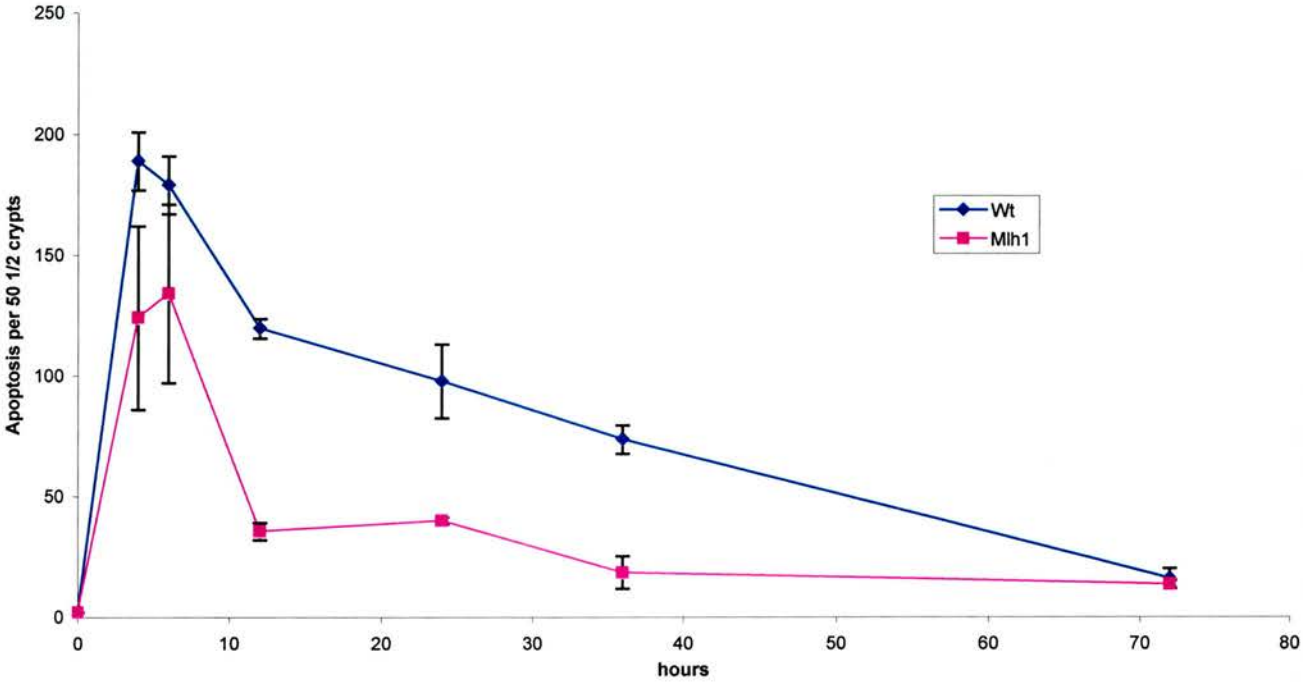
To determine if this represented a generic difference in the gene dependency of the apoptotic response to alkylating agents, this study was extended to temozolomide which produces a similar spectrum of lesions to NMNU (Denny *et al.,* (1994), see appendix 13.1)

Figure 4.2: Apoptosis at 6 hours following 100mg/kg temozolomide. Apoptosis was scored 6 hours following treatment with NMNU. Black bar, wild type mice; Open bar, *Msh2*$^{-/-}$ mice; Grey bar, *Mlh1*$^{-/-}$ mice; blue bar, *Mlh1*$^{+/-}$ mice; Yellow bar, *Pms2*$^{-/-}$ mice, Green bar, *Pms2*$^{+/-}$ mice At least three mice were used for each timepoint and error bars represent SD.



In contrast to results with NMNU, a significantly reduced apoptotic response at six hours following temozolomide treatment was seen in both the *Mlh1*$^{-/-}$ and *Pms2*$^{-/-}$ mice compared to wild type mice (Mann Whitney, *Mlh1*$^{-/-}$ mice (n=10) compared to wild type mice (n=10), p=0.002, Pms2 compared (n=8) to wild type mice, $p<0.001$). The peak induction of apoptosis in the murine small intestine following alkylating agents is from 4 to 6 hours (Toft *et al.,* 1999). However the apoptotic response extends over a 72 hours period, therefore to investigate the precise kinetics of the apoptotic response apoptosis was scored over this period.

Figure 4.3. Apoptosis scored at 4, 6, 12, 24, 36 and 72 hours following 100mg/kg temozolomide. Black line, wild type mice; Dotted line, *Msh2*$^{-/-}$, Pink line, *Mlh1*$^{-/-}$ mice. At least 3 mice were used for each timepoint and error bars represent SD.



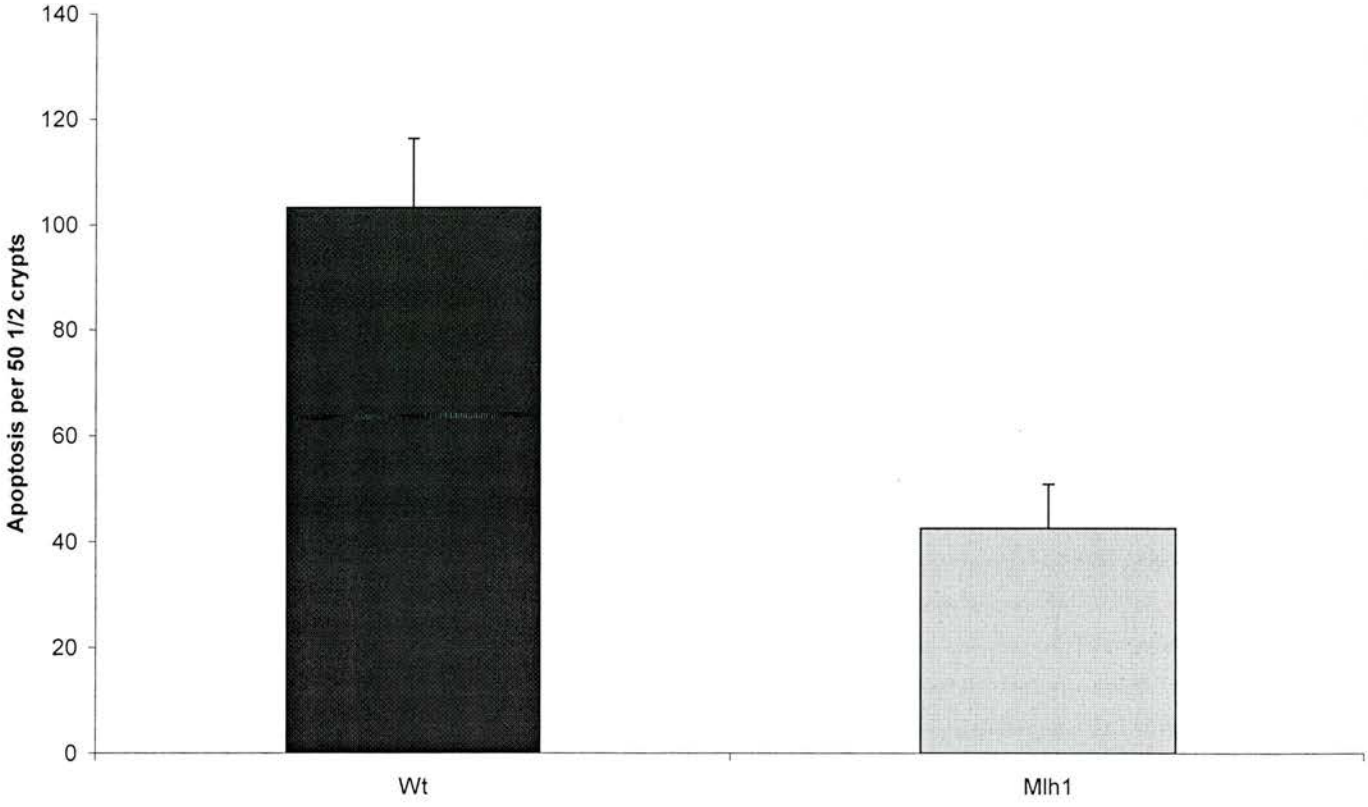
From the 72 hour timecourse, it was clear that *Mlh1* deficiency produces a marked reduction in the apoptotic response following temozolomide. At each of the six timepoints there was a significant reduction (Mann Whitney, p=<0.05) in the level of apoptosis compared to wild type mice.

4.1.3 Apoptosis following 10mg/kg of NMNU is *Mlh1* dependent.

The observed differences in the gene dependency of the apoptotic response was hard to reconcile with the hypothesis that both temozolomide and NMNU elicit apoptosis through the same mechanism, namely the production of the O6meG lesion (Tentori *et al.*, 1997, Bignami *et al.*, 2000). Even the spectrum of lesions they produce are very similar, although NMNU has been shown to produce a

higher percentage of O6meG lesions (Pieper 1995, Eisenbrand *et al.,* (1994), Goldmacher *et al.,* (1986) and Denny *et al.,* (1994) and see appendix 13.1 for lesion profile of temozolomide and NMNU). This, in conjunction with the slightly higher levels of apoptosis elicited by NMNU raised the possibility that the gene dependency of the MLH proteins at this level of DNA damage was sufficiently saturating to render undetectable any deficiency associated with *Mlh1*$^{-/-}$ deficiency. Thus the apoptotic response to a lower dose (10mg/kg) of NMNU was investigated.

Figure 4.4. Reduced apoptotic response in *Mlh1*$^{-/-}$ mice. Apoptosis was scored in 50 ½ crypts six hours following 10mg/kg NMNU treatment. Black bars, wild type mice; grey bars, *Mlh1*$^{-/-}$ mice. At least 3 mice were used for each timepoints and error bars represent SD.



After treatment with 10mg/kg NMMU, there was a significant reduction in the apoptotic response in *Mlh1*$^{-/-}$ mice (Mann Whitney, p=0.04). This argues very strongly that at the higher levels of NMNU apoptosis, the *Mlh1* dependence of this pathway is swamped, with apoptosis occuring in a *Mlh1* independent fashion. Whilst at low levels of apoptosis *Mlh1* is required for the normal apoptotic response.

4.1.4. No reduction in BrdU incorporation following temozolomide.

The replicative cycling hypothesis invokes futile cycles of repair. Therefore this leads to the prediction that as a consequence of these cycles of repair one would expect perturbation of cell cycle kinetics especially within S Phase. Agents such as gamma irradiation and 5-FU have been shown to produce a G1 arrest *in vivo* (Clarke *et al.,* 1995, Merrit *et al.,* 1995, Pritchard *et al.,* 1998). One approach to examine cell cycle kinetics *in vivo* is to investigate the level of BrdU incorporation during S Phase by exposing mice to BrdU *in vivo*. Cohorts of mice were therefore injected for two hours with BrdU before harvest. BrdU incorporation was then scored per 50 half crypts. Results represent the percent of live cells within the crypt labelled with BrdU.

Figure 4.5: Intestinal crypts stained with BrdU. Brown staining cells represent Brdu positive nuclei.

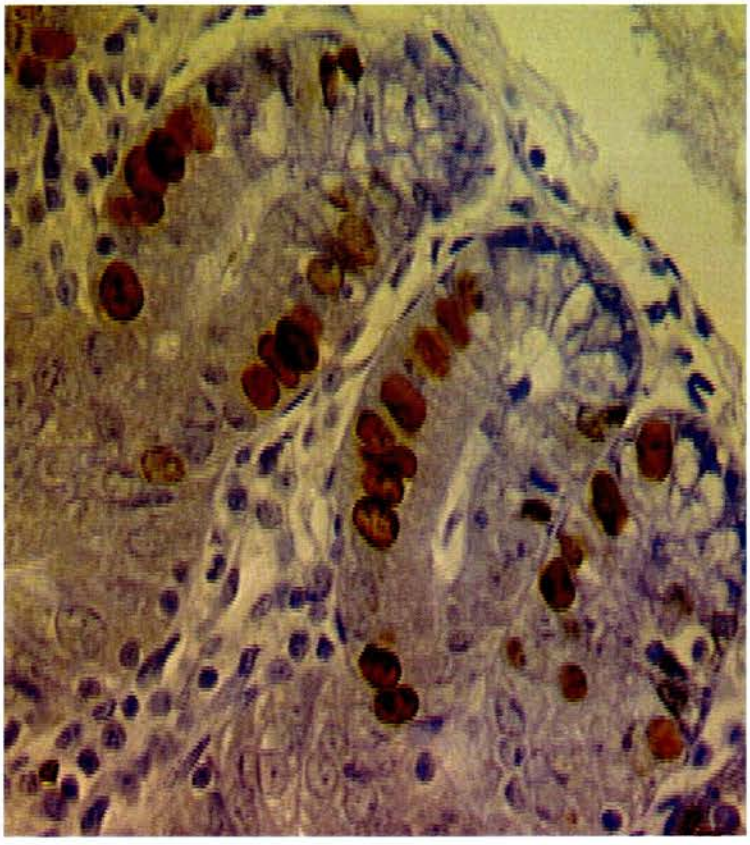
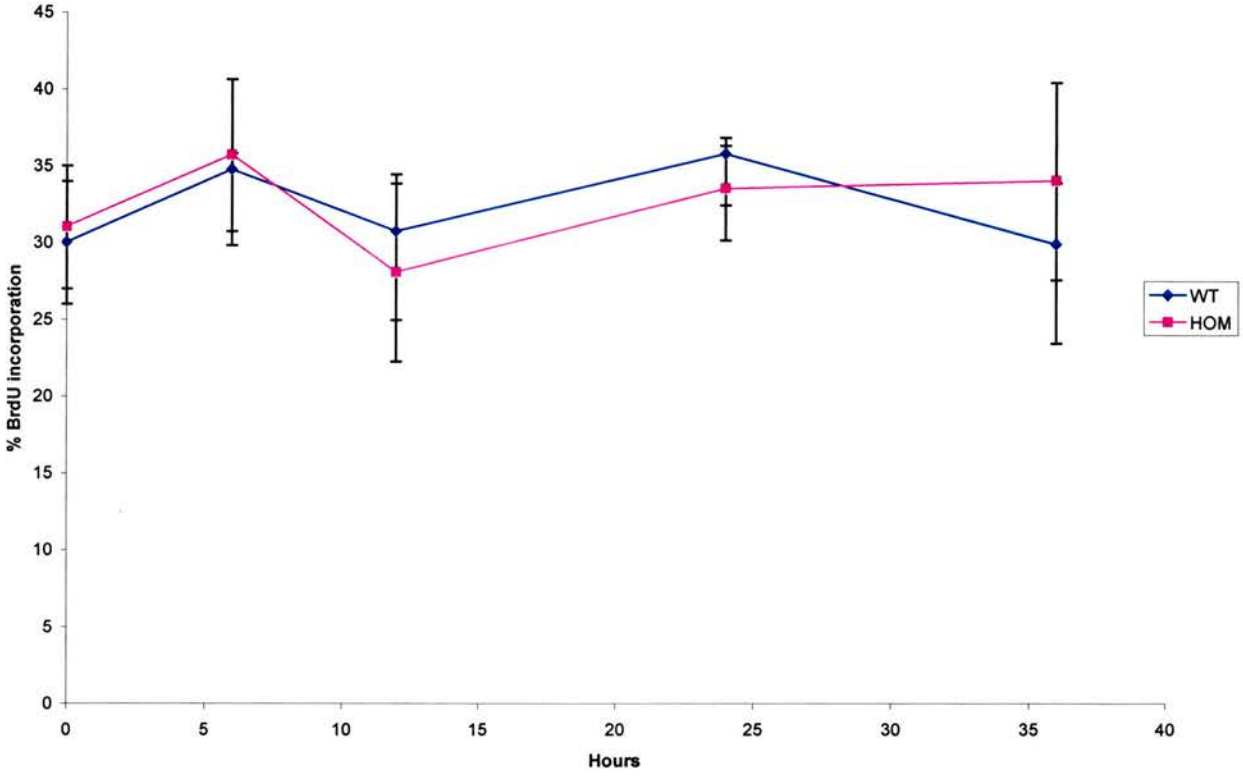


Figure 4.6. No reduction in BrdU incorporation over at 36 hour timecourse.

Mice were mock injected or injected for 6, 12, 24, 36 hours with 100mg/kg of temozolomide and injected with BrdU at 4, 10, 22 and 34 hours respectively. Black line, wild type mice; Pink line, *Mlh1*$^{-/-}$ mice. At least 3 mice were used at each timepoint and errors bars represent SD. No significant differences were seen at any of the timepoints compared to mock treated mice (Mann Whitney, $p<0.20$).



Unlike the scenarios reported for gamma irradiation and 5-FU (Clarke *et al.*, 1995, Pritchard *et al.*, 1998), there was not a significant reduction in BrdU labelling over the 36 hour timecourse in either wild type or *Mlh1*$^{-/-}$ mice.

4.1.5 Increased mutation following 100mgkg temozolomide in *Mlh1*$^{-/-}$ mice

Given that *Msh2*$^{-/-}$ deficiency produces a large increase in mutation frequency compared to wild type mice following temozolomide treatment (Toft *et al.*, 1999), the effect of *Mlh1* deficiency on mutation frequency at the *Dlb-1b* locus was then determined. This was of particular interest given there was a marked reduction in the apoptotic response in the *Mlh1*$^{-/-}$ nulls, yet no obvious perturbation of the cell cycle (as measured by BrdU incoropartion). Therefore this provided an excellent opportunity for examining the impact of the loss of an apoptotic pathway on long term mutation induction.

Figure 4.7. Intestinal wholemounts from (*Dlb-1a*, *Dlb-1b*) stained with Dlb1 peroxidase conjugate. Inset intestinal villus with white mutant clone dissected out from the intestinal wholemounts (large picture). The white mutant clones represent a loss of function mutation within the intestinal stem cell compartment of the *Dlb-1b* allele. Therefore as *Dlb-1a* is not expressed in the intestine a white mutant ribbon grows out.

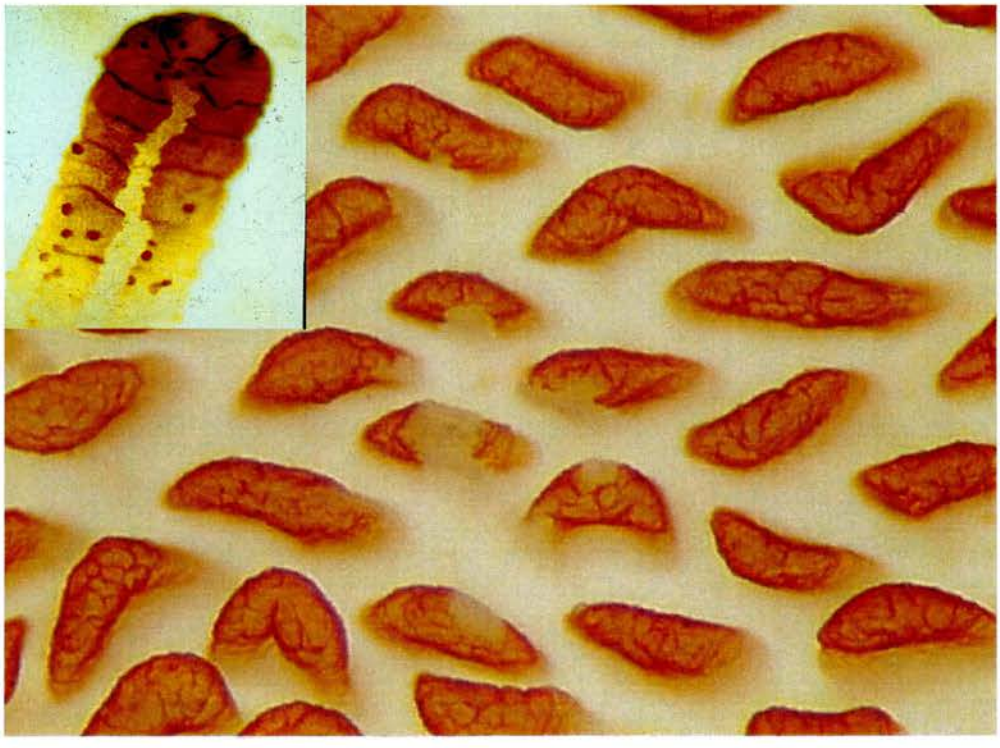
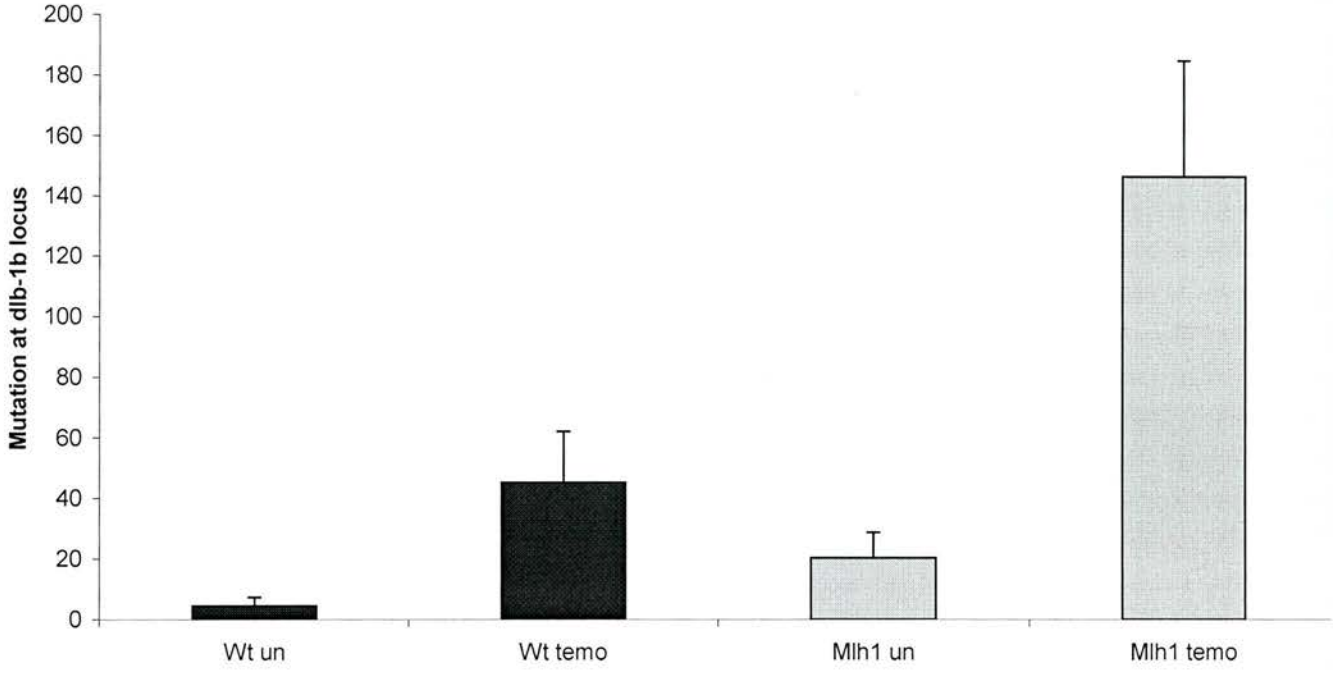


Figure 4.8 Increased mutation frequency at the *Dlb-1b* locus in *Mlh1*$^{-/-}$ mice compared to wild type mice. 8 week old male mice were injected with 100mg/kg of temozolomide for three weeks prior to harvest. Mutation frequency at the *Dlb-1b* locus was scored per 10000 villi. Black bars, wild type mice; Grey bars, *Mlh1*$^{-/-}$ mice. Mock treated mice, labelled un; mice treated with 100mg/kg temozolomide, labelled temo.



As expected *Mlh1*$^{-/-}$ mice (n=4) had significantly higher basal levels of mutation than wild (n=3) type mice (Mann Whitney, p=0.03). After treatment with 100mg/kg temozolomide there was a significant increase in mutation frequency in the treated *Mlh1*$^{-/-}$ mice (n=4) compared to treated wild type (n=8) (Mann Whitney, p=<0.001). Importantly this was not simply a reflection of the higher basal rates of mutation in untreated *Mlh1*$^{-/-}$ mice as the increase was much greater. On average the mock treated *Mlh1*$^{-/-}$ mice had 16 more mutations per 10000 villi than mock treated wild type mice, whilst temozolomide treated *Mlh1*$^{-/-}$ mice had 101 more mutations per 10000 villi than temozolomide treated wild type mice.

4.2. Discussion

Msh2, *Mlh1* and *Pms2* are all essential for MMR. Therefore it was assumed that they would all be essential for MMR-dependent apoptosis. However it has been shown here that for apoptosis following high levels of alkylation damage, they are not functionally equivalent. This has implications for the models of MMR-dependent apoptosis after alkylation damage.

The data produces two scenarios, one where there are relatively low levels of alkylation damage where functional MMR is required to elicit apoptosis and another where there are very high levels of alkylation damage where functional *Msh2* is required for apoptosis though not functional MMR (e.g. independent of *Mlh1* and *Pms2*). Consistent with this is that *Msh2* binds the methylation damage and thus is required for both damage recognition and apoptotic signalling.

Recently, dissociation of the roles that the MMR proteins play post alkylation damage was suggested by analyses of both the intracellular localisation and levels of Msh2 and Mlh1 prior to and following alkylation damage. Christmann and Kaina (2000) showed that following damage, levels of Msh2 increase dramatically in the nucleus through translocation of Msh2-Msh6 from the cytoplasm. However there was not a comparable increase in levels of Mlh1. Therefore one could envisage a situation where under normal circumstances Mlh1 and Msh2 are at relatively equal levels in the nucleus to facilitate functional MMR. However post damage there is a huge induction of Msh2-Msh6 (though not Mlh1) and thus Msh2-Msh6 can signal death independently of Mlh1 when there are high levels of damage. This Msh2-Msh6 signal is p53 dependent at six hours as there is virtually no apoptosis in the $p53^{-/-}$ mice.

This non-equivalence of the MMR proteins has been shown in other systems. For example MMR proteins are important in inducing somatic hypermutation, however when mutation spectra are examined in *Msh2*, *Mlh1* and *Pms2* nulls they are not the same (Reynauld *et al.*, 2000). The MutL homologues *Mlh1* and *Pms2*

function in meiosis whilst *Msh2* does not. This produces radically different phenotypes in the knockout mice as *Msh2* nulls are fertile, *Pms2* null females are fertile and all *Mlh1* nulls are sterile (Prolla *et al.,* 1997). Even in terms of MMR, *Mlh1* and *Pms2* null mice have different mutational spectra and frequency (Buermeyer *et al.,* 1999a). This is highlighted by the tumour predisposition of the *Pms2* nulls as none succumb to intestinal neoplasia unlike *Mlh1* and *Msh2* null mice (Prolla *et al.,* 1997).

One of the models of to explain how O6meG lesions elicit apoptosis is the replicative cycling model (described in 1.4.3) (Karran and Bignami, 1992). Briefly, this proposes cycles of futile repair that are dependent on MMR. It is these cycles of repair which are then thought to trigger apoptosis, possibly due to energy depletion or the persistence of strand breaks. However, at high levels of NMNU, it is shown here that there are normal levels apoptosis in both *Mlh1* and *Pms2* null mice. As the *Mlh1* and *Pms2* nulls are inefficient in MMR, it is highly unlikely that such futile cycling could occur, arguing against this model following high levels of alkylation damage.

Fishel, (1998, 1999) proposed that the MSH (Muts S Homologues) proteins act as an ADP-ATP switch (see 1.4.3). Recently Berardini *et al.,* (2000) showed that the Msh2-Msh6 heterodimer recognises O6meG lesions and stimulates the ADP-ATP switch. They speculated that if there were high lesional loads there may be a pathway that signals directly to apoptosis whilst a separate pathway signals for repair at lower levels of damage. Therefore it is possible (and indeed the data here implies) that this very high lesional pathway could be independent of Mlh1.

Another question raised here which relates to the replicative cyclng model of apoptosis is the failure to observe altered cell cycle kinetics (as measured by BrdU incorporation). Although not totally implicit in the replicative cyclig hypothesis, one would presume that these cycles of futile repair would lead to perturbations of the cell cycle. Therefore as no perturbations were discovered against this raises doubts on the replicative cycling model.

The finding that there is a significant *Mlh1* dependent increase in mutation frequency following 100mg/kg temozolomide treatment when there is significant *Mlh1* dependent apoptosis (in the absence of an arrest), is one of the clearest correlations between the failure to engage apoptosis and long term-term perpetuation of mutant cells *in vivo*. The next chapter will discuss how rarely the failure to engage apoptosis *in vivo* correlates with increased clonogenic survival and mutation . Therefore given that methylation damage is present in human gastrointestinal DNA (Hall *et al.,* 1991), this finding predicts that MMR deficiency will not only predispose to malignancy through failure to repair DNA but also through failure to engage apoptosis and delete damaged cells in the replicative and stem cell compartments of the intestinal crypts.

Chapter 5: Failure to engage apoptosis does not predict long-term survival in *p53* and *Msh2* deficient mice

5.0 Introduction

The previous two chapters have investigated the apoptotic response in the murine small intestine. This chapter examines the long term significance of this apoptosis using *Msh2*$^{-/-}$ and *p53*$^{-/-}$ mice.

It is widely considered that chemotherapy functions through the induction of apoptosis and that resistance is gained through loss of this apoptotic response (Lowe *et al.,* 1993,1994, Symonds *et al.,* 1994). The paradigm for this is the gene *p53*, which has been shown to be vital for the apoptotic response both *in vitro* and *in vivo* following a range of cytotoxic agents. *P53* is frequently lost during tumourigenesis, indeed the frequency of inactivation of *P53* may be much greater than first thought as a consequence of mutations in other genes in the *P53* pathway, as for example both upregulation of *MDM2* or loss of *ARF* affects *P53* expression (Prives and Hall, 1999). The seminal studies on the link between *p53*, apoptosis and chemotherapy were performed by Lowe *et al.,* (1993,1994) and Symonds *et al.,* (1994). They showed that a wide variety of anticancer drugs induced apoptosis in a *p53* dependent manner and that *p53* was important in suppressing tumour growth *in vivo*. Taking these findings together they argued that *p53* dependent apoptosis is vital for chemotherapy *in vivo*. However these studies did not prove a definitive link between loss of *p53* dependent apoptosis and chemotherapy, merely a correlation. Thus, Lowe *et al.,* (1993,1994) also showed there was a *p53* dependent arrest in response to many of these drugs, loss of which was also correlated with increased tumour volume in the *p53*$^{-/-}$ mice. Furthermore these studies made use of fibroblasts immortalised with either E1A or T antigen which differ from primary fibroblasts by their high sensitivity to apoptosis (Brown and Wouters 1999).

The generic relevance of these findings to spontaneous neoplasias is not yet clear. For example, in human colorectal cancer *P53* is frequently lost at the adenoma to adenocarcinoma transition (Vogelstein and Kinzler, 1996), yet in contrast to predictions from the Lowe and Symonds studies there are no differences in the levels of apoptosis between these stages of tumourigenesis (Fazelli *et al.,* 1997).

Correlations do not always exist between apoptosis and chemoresistance. In fact it is much easier to find instances where there is no link between apoptosis and chemotherapy. For example, Bearss *et al.,* (2000) showed that in mammary and salivary tumours arising in transgenic MMTV-*myc* and *MMTV-ras/ myc* transgenic mice, apoptosis was not important for the response to paciltaxel and doxorubicin. Normally these agents induce apoptosis, however in these tumours there was no induction of apoptosis but an induction of cell cycle arrest.

In order to determine the relevance of apoptosis to chemotherapeutic outcome, it is necessary to examine long term measures of survival. This can be done both *in vivo* and *in vitro* using clonogenic assays. Loss of *p53* has been shown on several occasions to be vital for the apoptotic response following gamma irradiation (Lowe *et al.,* 1993, Clarke *et al.,* 1993). However, outside of hematological systems where correlations between loss of apoptosis and increased survival do occur (Schmitt *et al.,* 2000, Griffiths *et al.,* 1997), loss of *p53* dependent apoptosis does not appear to correlate with increased survival. Brown and Wouters (1999) reviewed 10 different studies using *P53* deficient non-hematological tumour cell lines treated with gamma irradiation. Of these, 3 showed increased survival, 3 showed no difference whilst 4 reported increased sensitivity. Similarly, using the murine micro-colony assay of intestinal crypt survival *in vivo*, Hendry *et al.,* (1997) showed that *p53* deficiency caused complete abrogation of the immediate apoptotic response, yet this did not translate into an increase in long term survival.

Given the aim to tailor chemotherapy to the genetic profile of the tumour (Vogelstein *et al.,* 2000), it is of utmost importance that we truly understand the links between apoptosis and chemotherapeutic response. Clearly we are not yet in this position; as perhaps best exemplified by the debate that still continues over whether *P53* status is a good prognostic indicator of therapeutic outcome in colorectal cancer (Bosari and Viale 1995). Generally it appears that loss of *P53* is an indicator of poor responsiveness to therapy in colorectal cancer (e.g. Hamelin *et al.,* 1994, Hamada *et al.,* 1996, Benhattar *et al.,* 1996), however other studies have shown that loss of *P53* has a weak or even a beneficial effect upon therapy depending on tumour stage and location (e.g. Elsaleh *et al.,* 1999, Soong *et al.,* 1997, 1999, Poller *et al.,* 1997, Petersen *et al.,* 2001). If induction of apoptosis and chemotherapeutic success were

mutually dependent, one would have predicted a very clear correlation between *P53* status and prognosis.

In this study, the impact of loss of apoptosis (due to both *p53* and *Msh2* deficiency) upon long term crypt clonogenic survival *in vivo* is investigated with 3 different cytotoxic agents: Cisplatin, nitrogen mustard and N-Methyl-N-Nitrosourea (NMNU), each of which is briefly discussed below.

Cisplatin is a particularly effective chemotherapeutic against testicular and ovarian cancer. In ovarian cancer, resistance to cisplatin has been correlated with loss of the mismatch repair gene *MLH1* through promoter hypermethylation (Strathdee *et al.,* 1999). The putative cytotoxic lesion produced by cisplatin is a GG intrastrand crosslink which both *Msh2* and *p53* have been shown to bind (Duckett *et al.,* 1997, Karsparkova *et al.,* 2001). Cisplatin has been shown to be a potent inducer of both *Msh2* and *p53* dependent apoptosis in the murine small intestine *in vivo* (Toft *et al.,* 1999, Sansom *et al.,* 2001). Previous studies on clonogenic survival *in vitro* have produced contrasting results. Using the ovarian cell line A2760, loss of mismatch repair is associated with a small increase in resistance (1.5-3x) whereas *P53* deficiency was associated with a much larger increase (Brown *et al.,* 1997, Branch *et al.,* 2000). When Branch *et al.,* (2000) characterised a panel of MMR deficient and proficient colorectal cancer cell lines for resistance to cisplatin, MMR status did not influence survival. In contrast, Lin *et al.,* (2001) used isogenic HCT116 cell lines to show that *MSH2* deficiency caused an increase in survival whilst *P53* status made no difference to long term survival. In fact *P53* deficiency in the HCT116 cells failed to increase survival to a range of different agents including 6-thioguanine and etopside (see also Bunz *et al.,* 1999). We have previously analysed the response to cisplatin *in vivo* in the murine intestinal epithelium. In this context, *p53* deficiency causes complete abrogation of the immediate wave of apoptosis directly predicting an increase in clonogenic survival. Similar analysis showed $Msh2^{-/-}$ deficiency to weakly reduce the apoptotic response, predicting a more modest increase in survival.

Nitrogen mustard is one of the oldest chemotherpeutics still in clinical use (Hemminki 1994). It produces crosslinks in the DNA and is a potent inducer of apoptosis, implicating a *p53* dependent apoptotic response in the small intestine. However, Fan

et al., (1997) showed that *P53* deficient HCT116 cells treated with either nitrogen mustard or cisplatin were more sensitive than wild type isogenic HCT116 controls.

The alkylating agents MNNG, NMNU and temozolomide are thought to induce apoptosis through the production of the cytotoxic O-6 methyl guanine (O-6meG) lesion (Karran and Bignami, 1991). Duckett *et al.,* (1997) have shown that *Msh2*-Msh6 complex (MutS alpha) can bind this lesion. The alkylating agents temozolomide and MNNG have been shown to induce MMR dependent apoptosis in the murine intestine (Toft *et al.,* 1999). The last chapter discussed the importance of MMR following alkylation damage. The role of *p53* is much more debatable. Toft *et al.,* (1999) showed that *p53* deficiency caused abrogation of the immediate wave of apoptosis following the alkylating agents MNNG and temozolomide. As with irradiation, temozolomide also induced a delayed, reduced wave of *p53* independent apoptosis. In this case this was shown to be *Msh2* dependent. Duckett *et al.,* (1999) have also showed that Muts alpha was required for phosphorylation of *P53* post alkylation damage again suggesting a role for *P53* in signalling from alkylation damage. However the *p53* dependency for apoptosis following alkylation does appear to be cell type specific. For example, Hickman and Samson (1999) showed that apoptosis was only partially dependent on *P53* in the human lymphoblastoid TK line but totally dependent upon MutS alpha. Similarly Ochs and Kaina (2000) showed apoptosis following MNNG was *p53* independent in CHO cells.

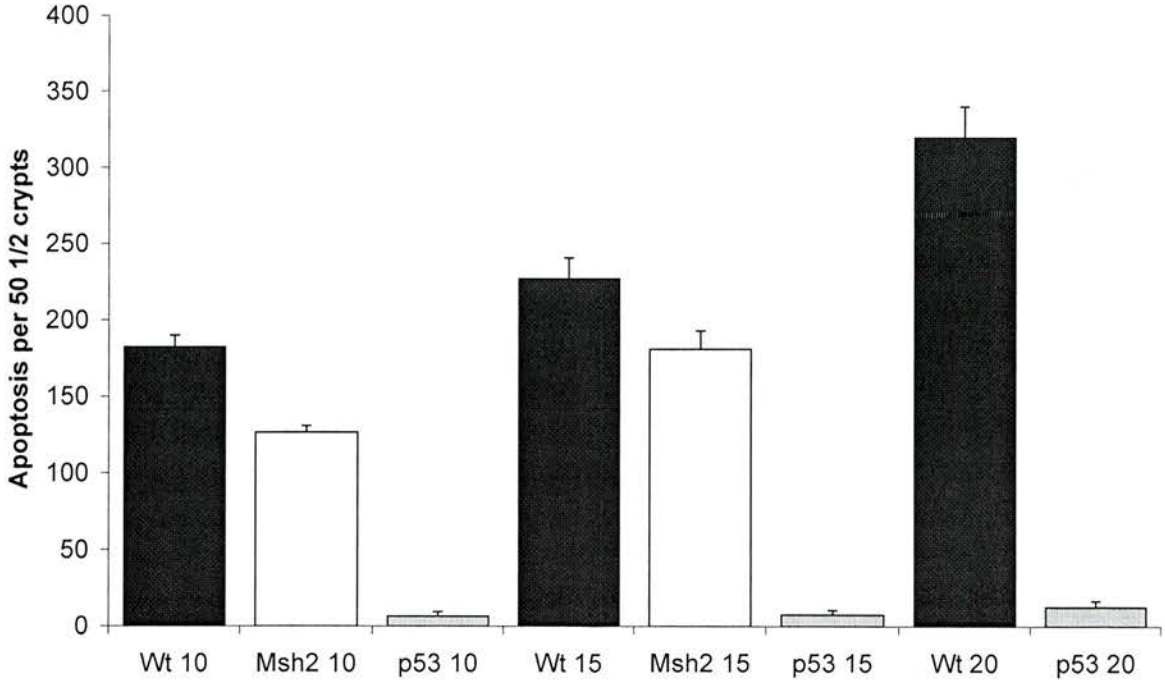
In order to further examine the relationship between apoptosis and chemotherapeutic outcome, and to define the roles of *Msh2* and *p53* in these processes, the apoptotic responses in $Msh2^{-/-}$ and $p53^{-/-}$ mice were investigated and compared to long term survival *in vivo* following exposure to cisplatin, nitrogen mustard and NMNU.

5.1 Results

5.1.1 Apoptosis following cisplatin damage

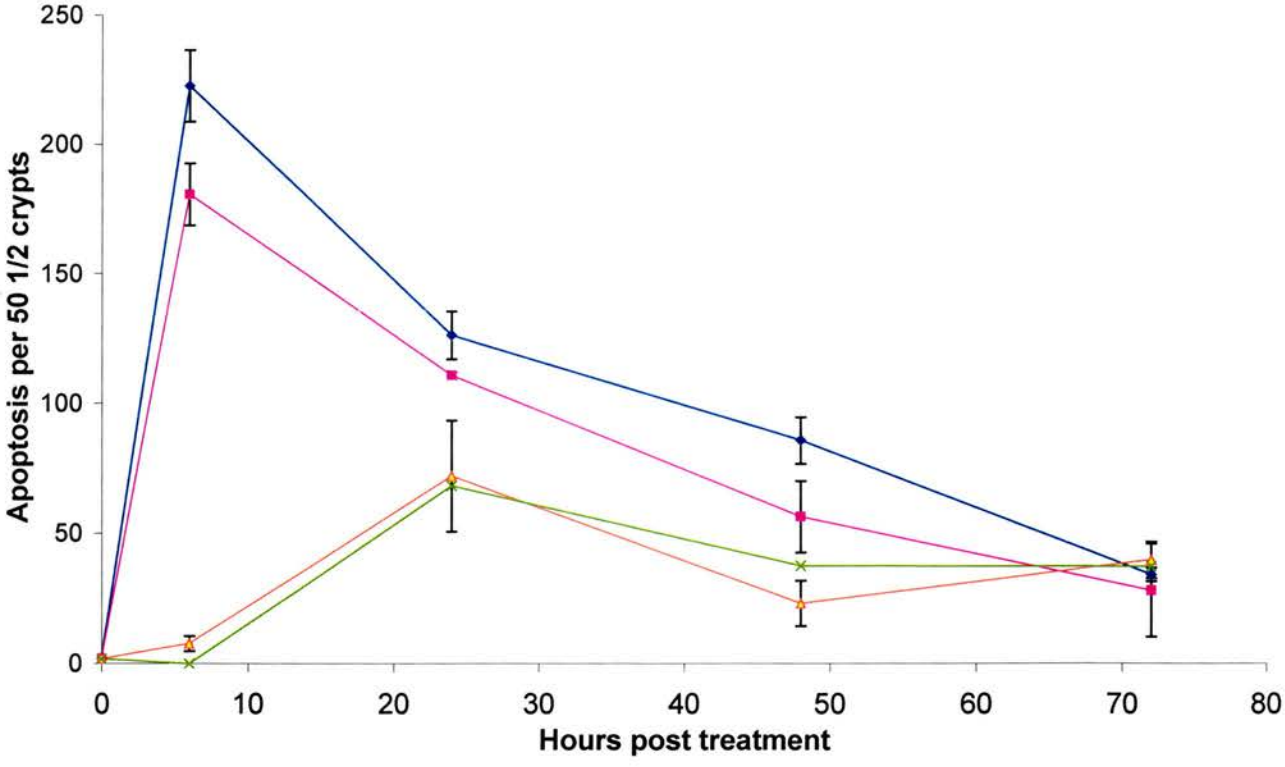
The apoptotic response to cisplatin at 10mg/kg, 15mg/kg and 20mg/kg of cisplatin at 6 hours following exposure was characterised (Figure 5.1). A dose dependent increase in apoptosis was seen in wild type intestinal enterocytes in response to cisplatin. *Msh2* deficiency caused a slight yet significant reduction in the apoptotic response and again there was a dose dependent increase. *P53* deficiency caused a complete abrogation of the apoptotic response at all 3 doses.

Figure 5.1: Apoptosis at scored 6 hours following 10mg/kg, 15mg/kg and 20mg/kg of cisplatin. Black bars, wild type mice; open bars, *Msh2*$^{-/-}$ mice; grey bars, *p53*$^{-/-}$ mice. At least 3 mice were used for every time point and error bars represent SD. Deficiency of p*53* led to loss of the apoptotic response at 6 hours at all doses compared to wild type mice (Mann Whitney, p=0.04) whilst *Msh2* deficiency caused a significant reduction in apoptosis at all doses used (Mann Whitney, p=0.04).



In order to investigate whether there was a delayed apoptotic response in the *p53* $^{-/-}$ and (*Msh2*$^{-/-}$, *p53* $^{-/-}$) mice, the apoptotic response was scored over a 72 hour period following cisplatin treatment. As has been reported with γ-irradiation (Clarke *et al.*, 1997, Merrit *et al.*, 1997) and temozolomide (Toft *et al.*, 1999) there was a delayed wave of *p53* independent apoptosis which peaked at 24 hours following cisplatin treatment. Previous reports by Gong *et al.*, (1999) argued that functional mismatch repair (MMR) was required to upregulate of *P73* and so mediate apoptosis in the absence of *p53* following cisplatin treatment. However after cisplatin treatment there was a delayed wave of apoptosis in the (*Msh2*$^{-/-}$, *p53* $^{-/-}$) mice indistinguishable from *p53*$^{-/-}$ mice (figure 5.2).

Figure 5.2: Apoptosis scored over a 72 hour timecourse following 15mg/kg of cisplatin. Black line, wild type; pink line, *Msh2*$^{-/-}$ mice; orange line, *p53*$^{-/-}$ mice; green line, (*Msh2*$^{-/-}$, *p53*$^{-/-}$) mice. In both *p53*$^{-/-}$ and (*Msh2*$^{-/-}$, *p53*$^{-/-}$) mice there was a delayed wave of *p53* independent apoptosis.



5.1.2.Clonogenic survival following cisplatin damage

In order to determine the effect of *p53* and *Msh2* deficiency on micro-colony survival, mice mutant for both genes were exposed to 15mg/kg of cisplatin (10mg/kg of cisplatin does not result in detactable crypt killing) Both $p53^{-/-}$ and ($Msh2^{-/-}$, $p53^{-/-}$) mice showed an increase in survival compared to wild type mice (Figure 5.4).

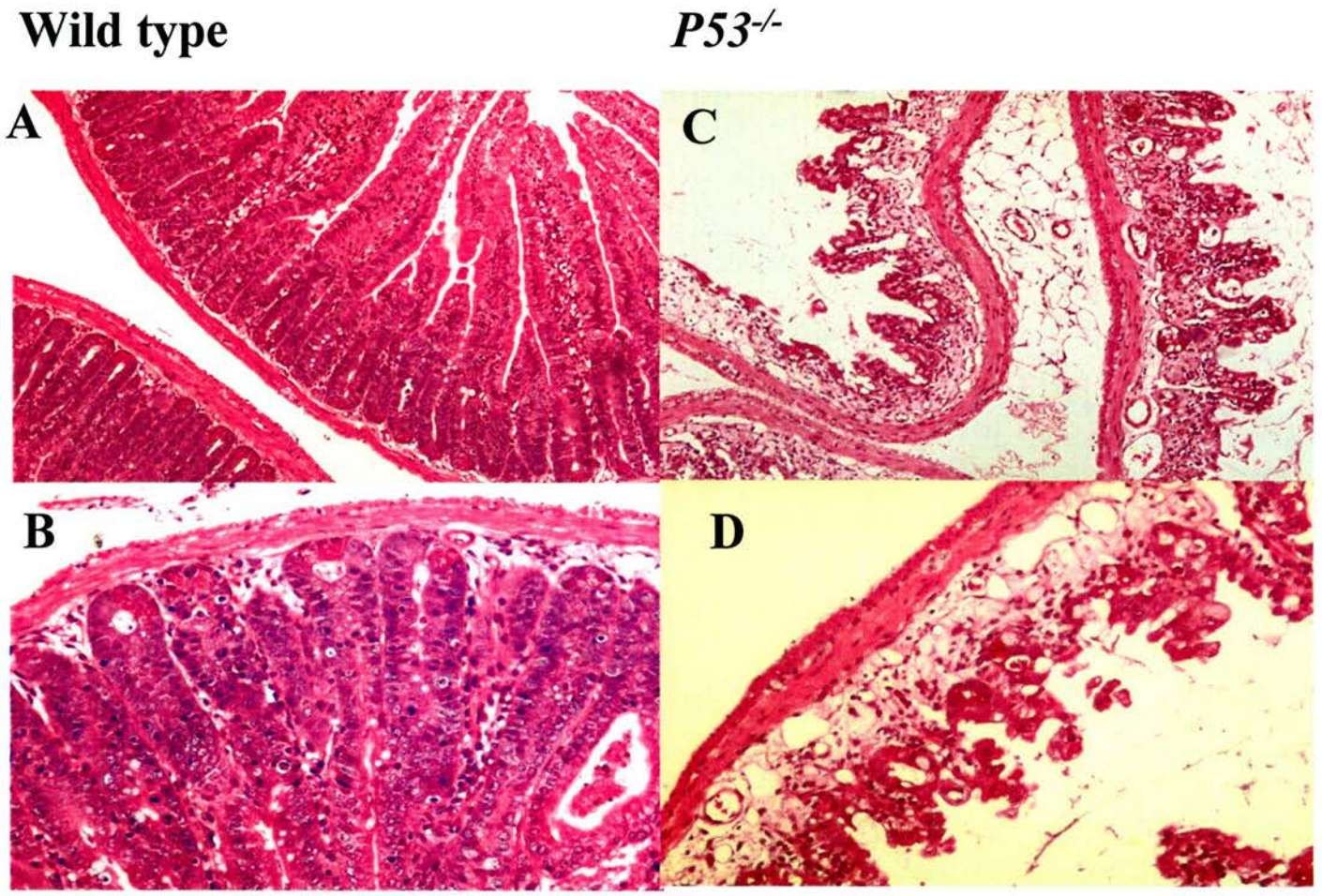
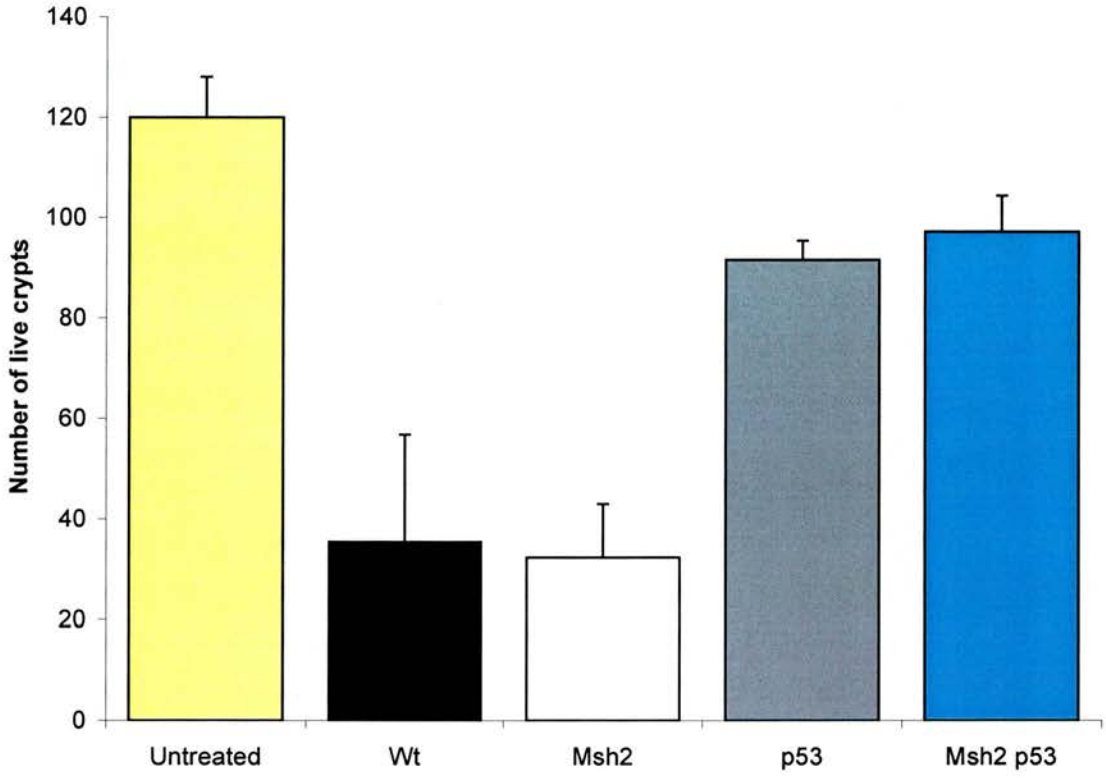


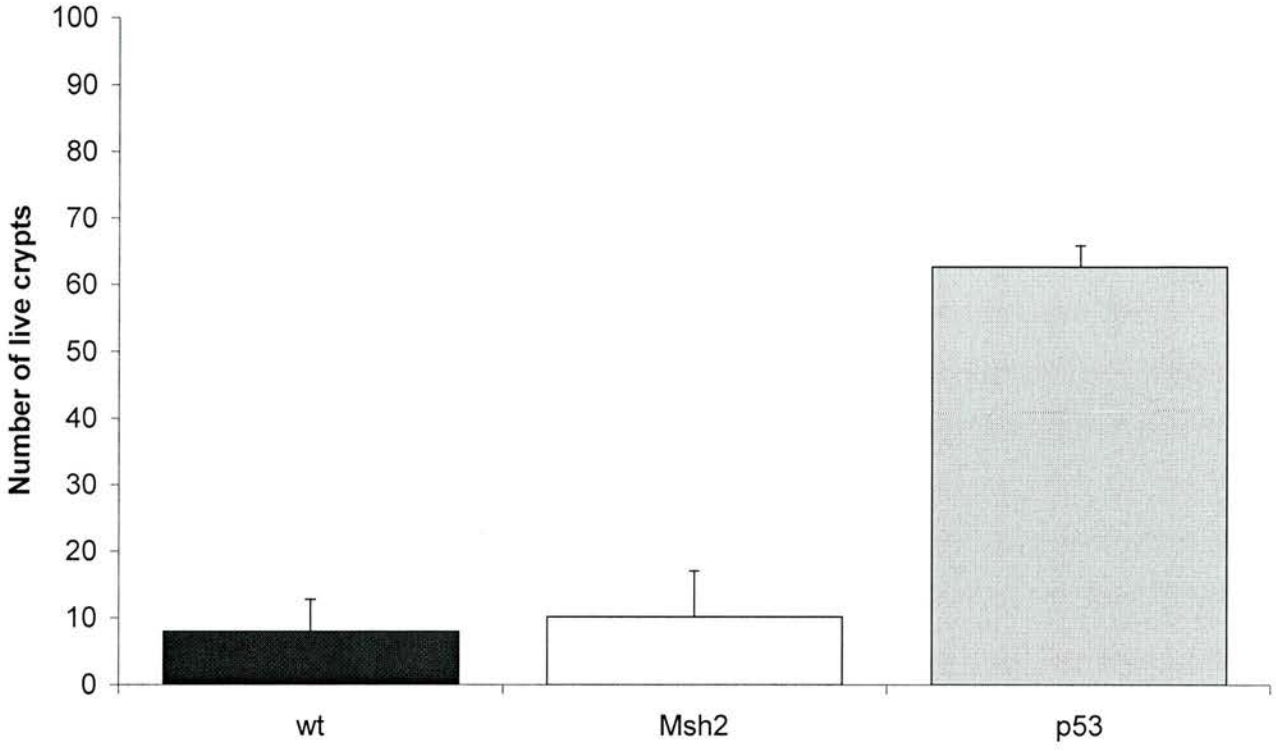
Figure 5.3 Clonogenic survival following 15 mg/kg cisplatin.
Pictures A (x 100) and B (x400) display a *p53* deficient mice exposed to cisplatin. Here the crypts are intact following 72 hours following cisplatin treatment. Pictures C (x 100) and D (x 400) display a wild type mouse 72 hours following cisplatin treatmetnt. As can be clearly seen, there are no intact crypts

Figure 5.4 Clonogenic survival at 15mg/kg of cisplatin as scored by the microcolony assay (Potten *et al.*, 1997). Striped bars, untreated mice; black bars, wild type mice; open bars, *Msh2*$^{-/-}$ mice; grey bars, *p53*$^{-/-}$; Blue bars, (*Msh2*$^{-/-}$, *p53*$^{-/-}$) mice. Both *p53*$^{-/-}$ and (*Msh2*$^{-/-}$, *p53*$^{-/-}$) mice showed increased clonogenic survival compared to wild type and *Msh2*$^{-/-}$mice (Mann Whitney, p=<0.04).



At the higher dose of 20mg/kg the extent of killing by cisplatin was increased with nearly total crypt death in the wild type and *Msh2*$^{-/-}$ mice. *P53* deficiency led to a marked increase in survival (Figure 5.4).

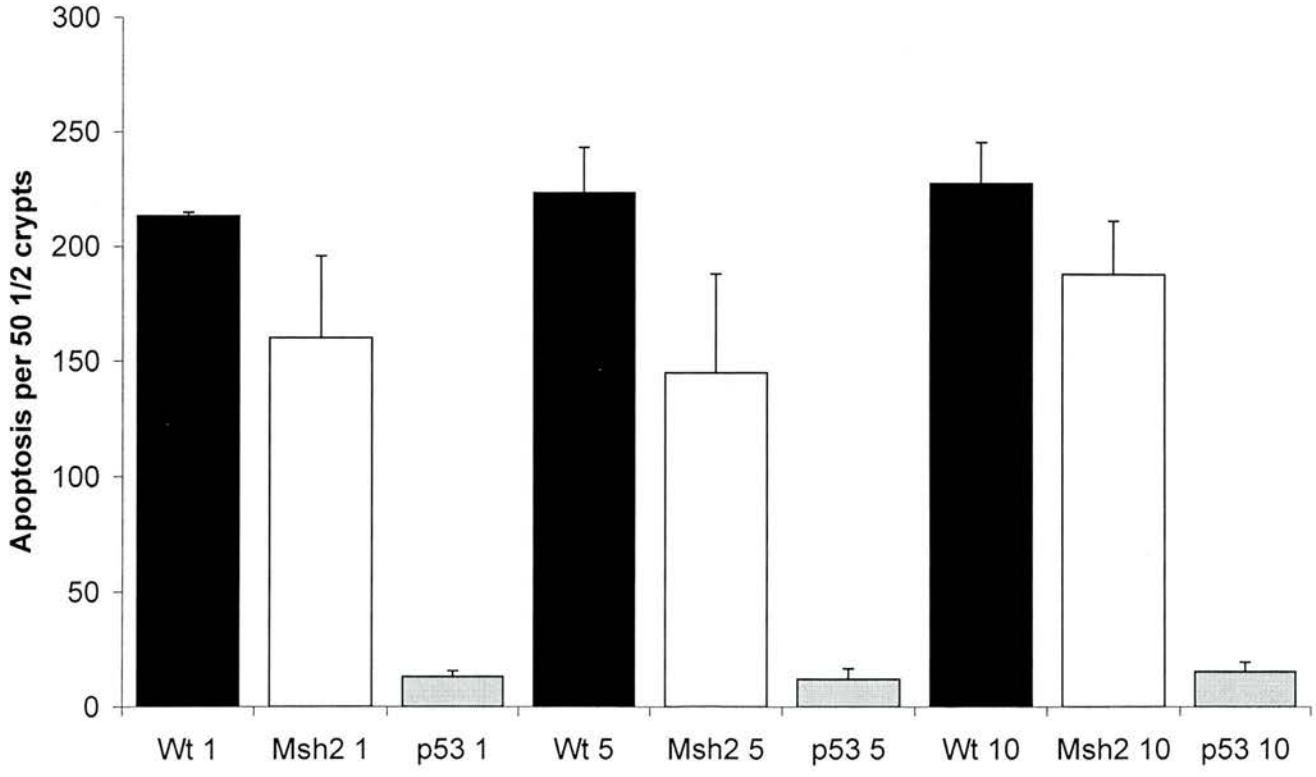
Figure 5.5 Microcolony survival at 20mg/kg of cisplatin. Black bars, wild type mice; open bars, *Msh2*$^{-/-}$ mice; grey bars, *p53*$^{-/-}$ mice. Again *p53*$^{-/-}$ mice showed increased clonogenic survival compared to wild type and *Msh2*$^{-/-}$mice (Mann Whitney, p=<0.04).



5.1.3. Apoptosis following Nitrogen Mustard.

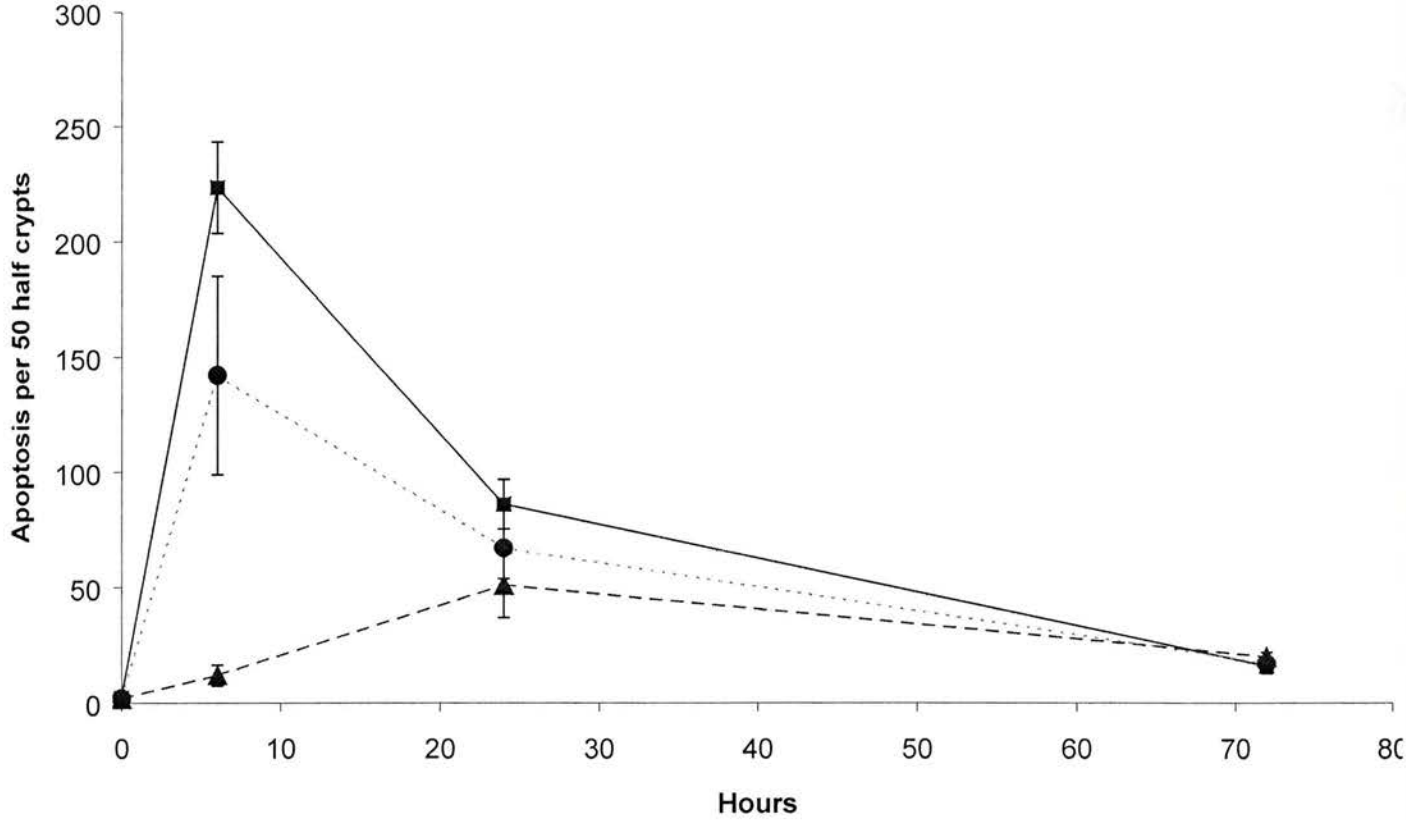
Apoptosis was scored at 6 hours in wild type, *Msh2*$^{-/-}$ and *p53*$^{-/-}$ mice following three different doses of Nitrogen Mustard. At the levels of Nitrogen mustard used, no dose dependency was observed in the apoptotic response (Figure 5.5). A small, significant reduction in apoptosis in *Msh2*$^{-/-}$ mice was seen at 1mg/kg and 5 mg/kg ($p=0.04$, Mann Whitney). No induction of apoptosis, above basal levels, was observed in the *p53*$^{-/-}$ mice

Figure 5.6: Apoptosis scored at 6 hours following 1 mg/kg, 5mg/kg and 10mg/kg of Nitrogen Mustard. Black bars, wild type mice; open bars, *Msh2*$^{-/-}$ mice; grey bars, *p53*$^{-/-}$ mice. At least 3 mice were used for every time point and error bars represent SD. *P53* deficiency led to loss of the apoptotic response at 6 hours to all dose (Mann Whitney, $p=0.04$) compared to wild types whilst *Msh2* deficiency caused a small yet significant reduction at 1mg/kg and 5mg/kg though not 10mg/kg.



In order to investigate whether there was a delayed apoptotic response in the *p53* $^{-/-}$ and mice, the apoptotic response was scored over a 72 hour period following Nitrogen Mustard treatment (Figure 2B). Once again there was a small delayed wave of *p53* independent apoptosis, which was highest at 24 hours following Nitrogen Mustard treatment.

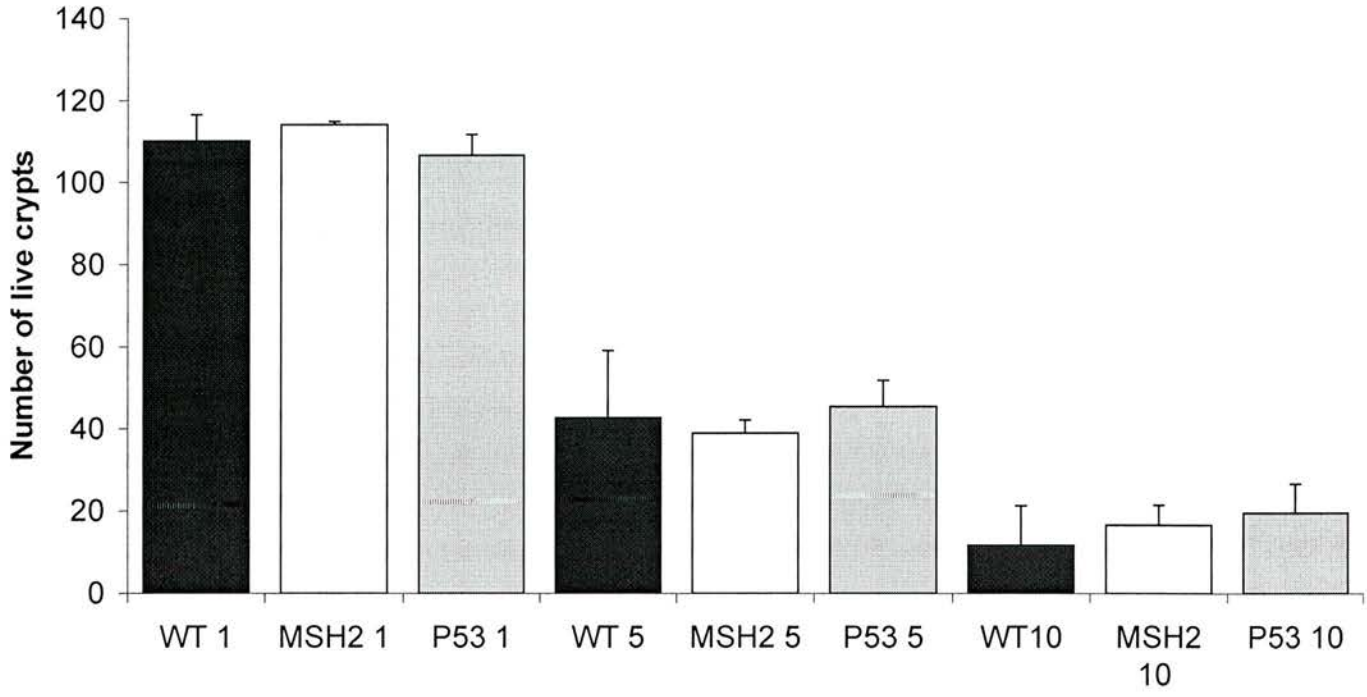
Figure 5.7:Apoptosis scored over a 72 hour timecourse following 5mg/kg of Nitrogen Mustard. Solid black line with square points, wild type; hashed line with circular points, *Msh2*$^{-/-}$ mice; hashed line with triangular points , *p53*$^{-/-}$ mice. In the *p53*$^{-/-}$ mice there is a delayed wave of *p53* independent apoptosis peaking at 24 hours



5.1.4.Clonogenic survival following Nitrogen Mustard.

Figure 5.8 shows the clonogenic survival as determined by the micro-colony assay following 1,5 and 10mg/kg Nitrogen Mustard. Despite the clear gene dependency of the apoptotic response no gene dependency was observed in crypt survival for either *Msh2*$^{-/-}$ or *p53*$^{-/-}$ mice.

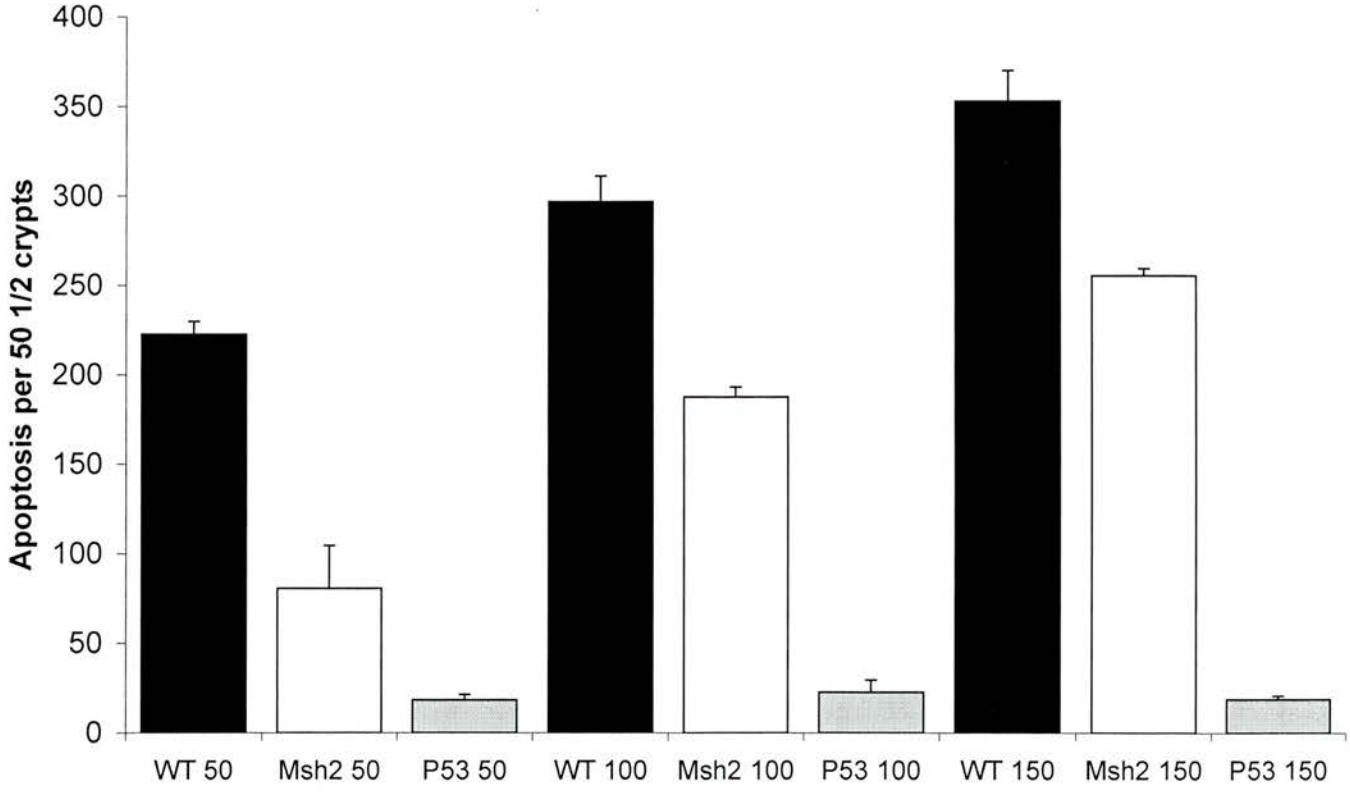
Figure 5.8. Microcolony survival at 1, 5 and 10mg/kg of Nitrogen Mustard. Black bars, wild type mice; open bars, *Msh2*$^{-/-}$ mice; grey bars, *p53*$^{-/-}$. There were no significant differences in clonogenic survival for any of the three genotypes (Mann Whitney, P>0.10).



5.1.5.Apoptosis following N-methyl-N-Nitrosourea (NMNU)

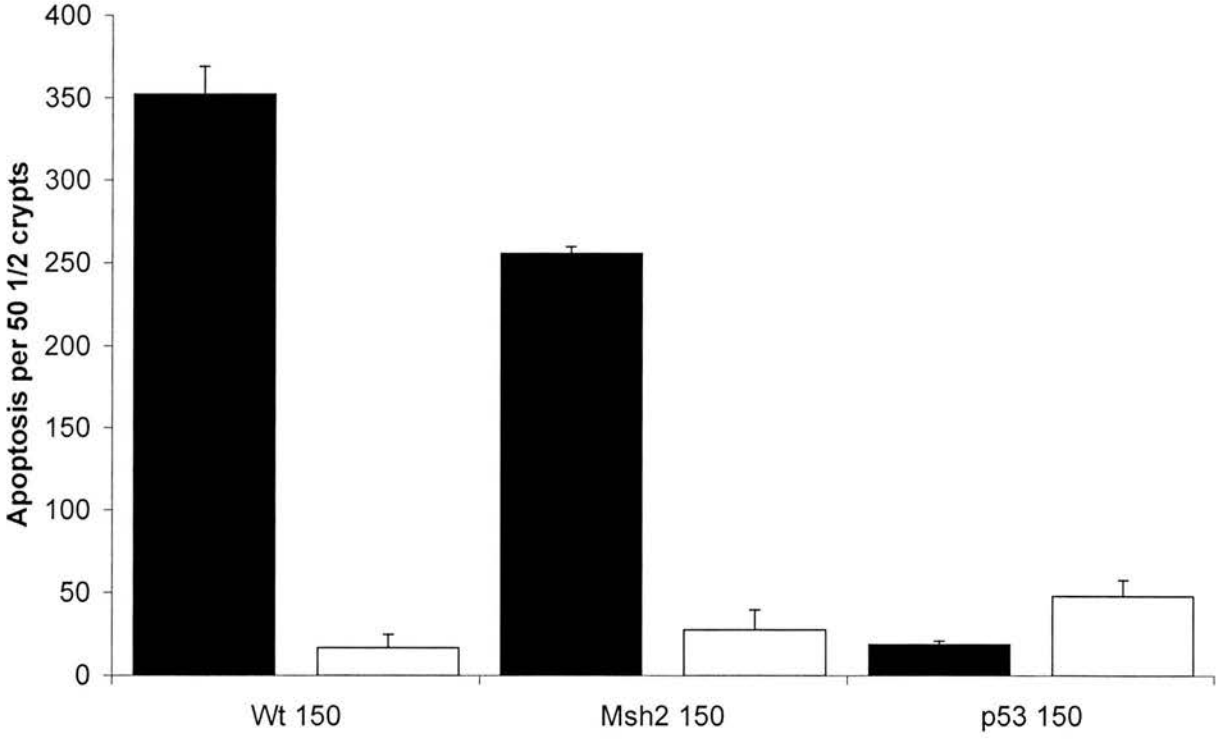
Apoptosis was scored at 6 hours following NMNU treatment. As with cisplatin, a dose dependency was observed in both wild type and *Msh2*$^{-/-}$ mice (figure 3A). *Msh2*$^{-/-}$ mice showed a significant reduction in the level of apoptosis at each dose. The extent of this reduction was much greater than that observed for both cisplatin and Nitrogen Mustard. Once again loss of *p53* causes abrogation of the apoptotic response at 6 hours.

Figure 5.9: Apoptosis scored at 6 hours following 50 mg/kg, 100mg/kg and 150mg/kg of NMNU. Black bars, wild type mice; open bars, *Msh2*$^{-/-}$ mice, grey bars, *p53*$^{-/-}$ mice. At least 3 mice were used for every time point and error bars represent SD. *P53* deficiency led to loss of the apoptotic response at 6 hours to all dose (Mann Whitney, $p<0.04$) compared to wild type mice whilst *Msh2* deficiency caused a significant reduction at all doses (Mann Whitney, $p<0.04$).



Apoptosis was scored at 72 hours to determine if there was a delayed apoptotic response (figure 5.9). At 72 hours, the apoptotic response was greatly reduced. However the *p53*$^{-/-}$ mice showed significantly higher levels of apoptosis (p=0.02 Mann Whitney), demonstrating a wave of *p53*-independent apoptosis. No evidence was seen for a delayed *Msh2*-independent wave of apoptosis.

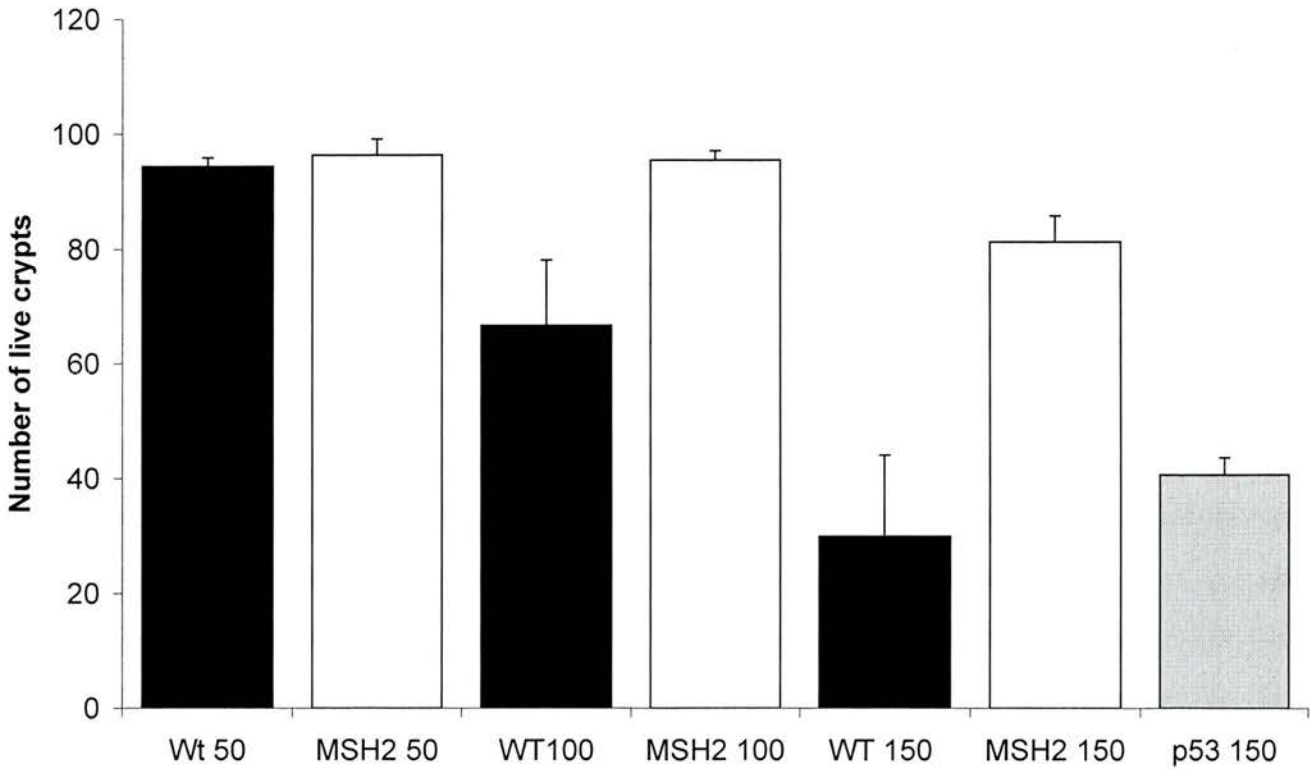
Figure 5.10: Apoptosis scores at 6 and 72 hours following 150mg/kg NMNU treatment. Open bars, 6 hours; Closed bars, 72 hours. In only the *p53*$^{-/-}$ mice there is a significant increase in apoptosis at 72 hours (Mann Whitney, p=0.04), corresponding to a delayed wave of *p53* independent apoptosis.



5.1.6.Clonogenic survival with NMNU

Previously NMNU has not been characterised with respect to crypt survival using the micro-colony assay. Therefore an escalating dose of NMNU was used in order to determine its impact upon crypt survival (figure 5.10). A dose of 150mg/kg of NMNU results in a high level of crypt death in wild type mice. In the absence of *Msh2* there was an increase in survival at this dose, but this was not observed in the absence of *p53*.

Figure 5.11: Clonogenic survival at 50, 100 and 150mg/kg of NMNU. Black bars, wild type mice; open bars, *Msh2*$^{-/-}$ mice, grey bars, *p53*$^{-/-}$. Doses of 50 and 100mg/kg do not produce high levels of crypt killing. 150mg/kg kills approximately 50% of crypts in wild type and *p53*$^{-/-}$ mice. *Msh2* deficiency causes a significant increase in crypt survival (Mann Whitney, p=0.02).



The table below summarises all apoptosis and survival data..

Table 5.12: Summary of apoptosis and clonogenic data. For apoptosis: single arrow pointing up, normal apoptotic response; single arrow pointing down, slight (though significant) reduction; 2 arrows down, large reduction in the apoptotic response, 3 arrows, virtually no apoptotic response. For clonogenic data: one arrow pointing down, normal survival, one arrow point up, increased survival.

	Apoptosis			**Clonogenic survival**		
	WT	*Msh2*	*p53*	Wt	*Msh2*	*p53*
Cisplatin	↑	↓	↓↓↓	↓	↓	↑
Nitrogen Mustard	↑	↓	↓↓↓	↓	↓	↓
NMNU	↑	↓↓	↓↓↓	↓	↑	↓

5.2.Discussion

The murine small intestine provides an excellent model for investigating the genetic dependency of apoptosis, longer term survival and mutation following DNA damage. Results from these assays contradict the simple model whereby loss of apoptosis is predicted to cause cells to persist long term and so increase the burden of mutation and predisposition to malignancy (reviewed in Sansom and Clarke 2000). For example, despite the well documented *p53* dependent apoptotic response of murine enterocytes, $p53^{-/-}$ mice do not have higher basal levels of mutation and only show increased levels of mutation following very high levels of gamma irradiation (Sands *et al.,* 1997, Clarke *et al.,* 1997). $P53^{-/-}$ mice also do not show increased clonogenic survival following gamma irradiation (Hendry *et al.,* 1997).

5.2.1.Genetic control of apoptosis

Following each of the three agents used there was complete abrogation of the immediate wave of apoptosis in the absence of *p53*. This highlights the role of *p53* as the central mediator of the apoptotic response in the murine small intestine to DNA damaging agents (Clarke *et al.,* 1994). Each of the three agents also induced a smaller, delayed wave of *p53* independent apoptosis. As the micro-colony assay is performed at 72 hours, it would be unlikely that this delayed apoptosis would account for crypt death. Therefore this predicts that loss of the apoptotic response in $p53^{-/-}$ mice should cause an increase in clonogenic survival following all three of the agents.

After treatment with NMNU, *Msh2* deficiency leads to a reduction in the apoptotic response. This is consistent with previous studies using temozolomide and MNNG, highlighting the role of MMR in signalling apoptosis from O^6 methylguanine lesions (Toft *et al.,* 1999). *Msh2* deficiency also led to a reduction in the apoptotic response to both cisplatin and nitrogen mustard. In the case of nitrogen mustard, this reduction in apoptosis is unexpected as there is no existing evidence that MMR recognises this type of damage. This could reflect a compromised generic apoptotic response in the MMR deficient mice, consistent with studies that have shown MMR to be important after a range of DNA damage including oxidative damage, ionising radiation, alkyaltion damage, cisplatin, and 6 thio-guanine (De Weese *et al.,* 1998, Fink *et al.,*

1998). Another explanation for the reduction of apoptosis in *Msh2*$^{-/-}$ mice is that *p53* upregulation and phosphorylation is partially dependent on functional MMR (as has been shown for both cisplatin and MNNG)(Lin *et al.,* 2001, Duckett *et al.,* 1999), such that absence of MMR could lead to a reduction in the *p53* dependent wave of apoptosis.

Gong *et al.,* (1999) have previously shown that following cisplatin treatment of HCT116 cells, the *p53*-independent apoptotic response was mediated by p73 and furthermore that this was MMR dependent. This leads to the prediction that (*Msh2*$^{-/-}$, *p53*$^{-/-}$) mice should lose their *p53* independent apoptotic wave in response to cisplatin, a phenomenon demonstrated by Toft *et al.,* (1999) following temozolomide treatment. However, it was shown here that following cisplatin treatment the (*Msh2*$^{-/-}$, *p53*$^{-/-}$) mice had an identical apoptotic response to *p53*$^{-/-}$ mice. This raises two possibilities, first, that p73 is still induced in the (*Msh2*$^{-/-}$, *p53*$^{-/-}$) mice independently of MMR or, second, that p73 is not necessary for this delayed wave of apoptosis.

5.2.2. Genetic control of clonogenic survival

The results presented here clearly show that the ability to engage apoptosis does not directly predict long term survival. Two very different scenarios are reported:

1)Where abrogation or reduction of the immediate apoptotic response correlates with increased long-term survival

Following cisplatin treatment, *p53* deficiency resulted in diminished apoptosis and increased crypt survival. Previously Pritchard *et al.,* (1998) have shown increased cellular survival within *p53*$^{-/-}$ intestinal crypts surviving 4 days post treatment with 400mg/kg 5-FU. However this did not correspond with loss of apoptosis as no increase was observed in the levels of apoptosis in wild type intestinal enterocytes between crypt killing and non-killing doses.

Therefore the data presented here is the first demonstration *in vivo* of increased crypt survival correlating with loss of a *p53*-dependent apoptotic response. The data is supported by the studies of Branch *et al.,* (2000) in ovarian cell lines where *P53* is a

major component of cisplatin resistance. These results predict that cisplatin would kill most effectively in a $P53^{+/+}$ environment. In this respect it is interesting that the ONYX 015 adenovirus, which replicates specifically in and kills $P53^{-/-}$ cells, has been shown to work well in combination with both 5FU and cisplatin. This suggests that ONYX 015 targets the $P53^{-/-}$ cells whilst the cytotoxic drugs targets the $P53^{+/+}$ cells (Khuri *et al.,* 2000). It is also relevant that testicular cancers are very sensitive to cisplatin treatment and very rarely show *P53* mutations (Fleischaker *et al., 1994*, Lutzkar and Levine 1996). Indeed testicular cancers have been shown to display very high levels of wild type *P53* following chemotherapy (Lutzkar and Levine, 1996) and the rare cases of resistance to cisplatin have been associated with loss of *P53* (Houldsworth *et al.,* 1998, Eid *et al.,* 1997).

Similar results were obtained following NMNU treatment of $Msh2^{-/-}$ mice, where there was also a reduced apoptotic response and increased clonogenic survival. This is consistent with previous data where loss of mismatch repair caused increased survival *in vitro* in ES cells following temozolomide and increased mutational burden *in vivo* following temolozomide, MNNG and NMNU (Andrew *et al.,* 1998, Toft *et al.,* 1999, Sansom *et al.,* 2001). This data therefore supports the paradigm of failed apoptosis leading to an increase in survival and therefore a higher burden of mutation burden.

Therefore it has been shown that loss of *p53* or *Msh2* can confer resistance *in vivo*. In the case of cisplatin, this would directly predict that *p53* status would be critical to therapy.

2) Where Reduced or abrograted of the apoptosis does not correlate with increased survival

In only 2 / 6 of experiments performed here did loss of apoptosis correlate with survival. *Msh2* deficiency did not lead to increased survival following either Nitrogen Mustard or cisplatin, despite a small reduction in the apoptotic response. Previous reports have strongly associated loss of MMR with cisplatin resistance in ovarian tumours. One explanation for our failure to observe increased survival (consistent with the previous chapter) may be that saturating levels of DNA damage may have been used, which could have obscured the impact of MMR deficiency. This is unlikely as it has previously shown that *Msh2* deficiency does not cause increases in mutation following low levels of cisplatin exposure (Sansom *et al.,* 2001). Furthermore it has shown here that the apoptotic response was not saturated at 15mg/kg, the high dosage used for the micro-colony assay.

The studies reported here have been performed in normal intestinal enterocytes. It remains possible that the reliance upon functional MMR markedly differs between normal and neoplastic cells. Pertinently, Strathdee *et al.,* (2001) have shown that mouse embryonic fibroblasts deficient for *Msh2* still undergo a G2 arrest following cisplatin treatment in marked contrast to MMR deficient tumour cell lines which lose this checkpoint (Brown *et al.,* 1997).

In fact, clonogenic survival appears to be very cell line and context dependent, as Lin *et al.,* (2001) show that *P53* does not affect survival in colorectal cell lines following cisplatin treatment in contrast to the study here and by that of Branch *et al.,* (2000). Probably the best study highlighting the importance of cellular context for apoptosis and survival was performed by Schmitt *et al.,* (2000). Here, using primary lymphomas, apoptosis and *Bcl-2* status was shown to be important for multidrug resistance and long term survival, however when these lymphomas were cultured *in vitro*, *Bcl-2* status was no longer important for clonogenic survival.

Deficiency for *p53* did not lead to increased survival following either Nitrogen Mustard or NMNU. Thus it is clear that those cells that escape the immediate wave of apoptosis do not survive long-term. This may help explain the apparent confusion

over the role of *p53* following alkylating agents, as although *p53* is important for the immediate wave of apoptosis this does not translate into long term survival. Thus, in terms of final outcome (survival) the results here concur with those of Hickman and Samson (1999), despite their failure to observe *P53* dependent apoptosis.

The findings also concur with previous studies of the effect of *p53* deficiency following γ-irradiation, where there was no increase in survival; and after 5FU damage, where increased survival was independent of apoptosis. Overall (including the 3 agents here) loss of *p53* dependent apoptosis can only potentially explain increased survival in 1/5 agents used *in vivo* (Pritchard *et al.,* 1999, Potten 1997).

One remaining question relates to the fate of those cells which do not engage apoptosis but do not survive over the long term. Four possibilities arise:
The first is delayed apoptosis. Delayed, *p53* independent apoptosis does occur but at greatly reduced levels compared to the immediate (6 hours) apoptotic response. The crypt replenishes itself from stem cells over a 72 hour period. Thus if all the stem cells and clonogenic cells die, three days later the crypt will be dead (reviewed in Potten 1990 and Potten *et al.,* 1997). However as the micro-colony assay is taken 72 hours after cytotoxic agent, delayed death occurring at 24-48 hours would not result in crypt death by the cessation of the assay. Although delayed apoptosis could affect survival at later times, it cannot be used to explain the death of crypts at 72 hours. Delayed apoptosis has been associated with large apoptotic bodies derived from cells dying out of G2/M of the cell cycle (Merrit *et al.,* 1997). Similar large apoptotic bodies were observed, coinciding with the onset of delayed *p53* apoptosis (24 hours and later). However these were not restricted to the *p53* deficient animals with all wild type and mismatch repair deficient mice also exhibiting these large apoptotic bodies

The second possibility is permanent arrest or senescence. This mechanism was invoked by Pritchard *et al.,* (1999) to explain the increased survival in the $p53^{-/-}$ animals after 5 FU treatment. If there is permanent arrest of stem cells and clonogenic cells after damage, the crypt would not become repopulated with cells and be killed 72 hours after insult. One of the difficulties with evaluating this mechanism is the lack of good markers for stem and clonogenic cells, which makes it impossible to

accurately determine the nature of these cells that are in permanent arrest (for a review see Potten 1998). Therefore, it is currently not possible to determine the contribution made by this mechanism to the loss of clonogenic potential.

The third possibility is necrosis. However no evidence of necrosis was observed during these studies nor was any sign of inflammation that would be associated with it.

The final possibility is deletion of otherwise viable crypt cells occurs as a consequence of loss of endothelial cells, which has been reported to occur following high levels of γ-irradiation (Paris *et al.,* 2001). The majority of the data presented here supports this argument that failure to engage apoptosis in the intestinal enterocytes is not a reliable indicator of survival. However the observation reported here that loss of gene dependent apoptosis (e.g. *p53* deficiency and cisplatin) in the intestinal crypts can correlate with survival, argues that the importance of endothelial apoptosis may be agent type specific.

Whatever the mechanism of the loss of these cells, these studies highlight that our assumptions concerning apoptosis and survival need to be re-addressed. Several studies have suggested that in colorectal cancer loss of apoptosis is important for tumour progression (Bedi *et al.,* 1995) with tumours showing lower rates of apoptosis compared to the normal epithelium. Furthermore studies using ($Apc^{Min+/-}$, $p53^{-/-}$) mice with intestinal adenomas show there is very little induction of apoptosis following radiation compared to ($Apc^{Min+/-}$, $p53^{+/+}$) adenomas (Fazelli *et al.,* 1998). If the simple hypothesis (that loss of apoptosis directly results in increased survival) is accepted then the prediction of these studies is that irradiation would be ineffective in treating *P53* deficient colorectal cancer. However γ-irradiation remains one of most clinically effective treatment for colorectal cancer, a scenario which is far more consistent with the data presented here on clonogenic survival which argues that *p53* status can be irrelevant to long term survival.

Taken together, our findings show that apoptosis cannot be used to directly predict long-term survival. Other studies have shown survival can vary from cell line to cell

line and from *in vitro* to *in vivo* (Brown and Woulters 1999, Schmitt *et al.,* 2000). In fact, predicting survival on the basis of gene dependent apoptosis *in vivo* appears to be virtually impossible, highlighting the difficulties in extrapolating system based responses from individual cellular responses. Interpreting these relationships represents a major challenge for our understanding of systems physiology. This has particularly important implications for the potential of gene based therapies which aim to restore apoptotic activity and furthermore argues that screens which score survival rather than the ability to induce apoptosis or arrest may be much better markers of prognostic success.

Chapter 6: Heterozygosity for *p53* promotes microsatellite instability on an *Msh2* deficient background.

6.0 Introduction.

Given that the acquisition of genomic instability in colorectal cancers has been associated with two separate pathways: a MSI MMR pathway and an aneuploid p53 pathway (see below), *Msh2*$^{-/-}$ and *p53*$^{-/-}$ mice were intercrossed to characterise whether there was an acceleration of neoplasia in these mice. (Cottu *et al.,* 1996; Lengauer *et al.,* 1997).

The first of these two pathways is characterised by defects in components of the DNA mismatch repair pathway, for example by *Msh2* deficiency. The primary manifestation of such loss is an increase in MSI (Liu *et al.,* 1995), presumed to arise as a direct consequence of failed DNA repair. The observation of a mismatch repair-dependent G2 cell cycle checkpoint (Hawn *et al.,* 1995) is consistent with such a role. However, mismatch repair proteins may also exert a protective effect by mediating the deletion of cells which bear DNA damage, as functional *Msh2* has been shown to confer sensitivity to alkylating agents both *in vitro* (De Wind *et al.,* 1995) and *in vivo* (as shown in chapter 4 and 5, Toft *et al.,* 1999).

The majority of colorectal cancers show no evidence of MSI. However, these cancers frequently show gross genomic instability (Reichmann *et al.,* 1981). Several lines of evidence suggest that defects in *p53* are associated with this form of instability. First, alterations in *p53* are correlated with the divergence of aneuploid sub-clones in colorectal cancers (Carder *et al.,* 1993). Second, cells become permissive for DNA amplification when *p53* is lost (Yin *et al.,* 1992). Third, cells derived from *p53* null mice display elevated levels of chromosomal instability (e.g. Harvey *et al.,* 1993). The role of *p53* is however not limited to preventing chromosomal instability, as *p53* transcriptionally regulates a number of genes associated with cell cycle arrest, apoptosis and DNA repair. Furthermore, the *p53* protein has been shown to directly interact with DNA, both through a 3'5' exonuclease activity (Mummenbrauer *et al.,* 1996) and through direct binding of the C-terminal domain to DNA mismatches (Lee *et al.,* 1995, Dudenhoffer *et al.,* 1998, Degtyareva *et al.,* 2001). P53 has therefore been characterised to mediate a number of functions which may have direct tumour suppressor activity. Some of these activities have been shown to be *p53* gene dose dependent (Clarke *et al.,* 1993, 1994 Rafferty *et al.,* 1996). It is therefore perhaps not surprising that evidence has been produced which suggests heterozygosity for *p53* may predispose to neoplasia (Venkatachalam *et al.,* 1998).

Recently evidence has emerged of a close relationship between *p53* and *Msh2* following DNA damage, most notably that MMR-mediated apoptosis is mediated through *p53* (Toft *et al.,* 1999) and that *p53* becomes phosphorylated in an MMR-dependent fashion after alkylation damage (Duckett *et al.,* 1998). To study the effects of *p53* and *Msh2* deficiency upon neoplasia, several murine strains have been produced which bear mutant alleles of *Msh2* (De Wind *et al.,* 1995; Reitmair *et al.,* 1995 and 1996a) and *p53* (Donehower *et al.,* 1992; Clarke *et al.,* 1993; Lowe *et al.,* 1993). Mice null for *p53* are viable, although a significant proportion of female $p53^{-/-}$ mice die during embryogenesis (Sah *et al.,* 1995; Armstrong *et al.,* 1995). Surviving animals rapidly develop tumours, predominantly thymic lymphomas with a smaller proportion succumbing to sarcomas (Donehower *et al.,* 1992; Purdie *et al.,* 1994). $p53^{+/-}$ heterozygote mice develop both lymphomas and sarcomas in approximately equal ratios, but with a longer latency period when compared to homozygote mice (Purdie *et al.,* 1994). Mice null for *Msh2* develop lymphomas with a peak incidence at 2-3 months of age (De Wind *et al.,* 1995; Reitmair *et al.,* 1995 and 1996). Of those animals surviving past 6 months of age, 70% develop intestinal neoplasms (Reitmair *et al.,* 1996) and 7% develop skin neoplasms analogous to those of the Muir-Torre syndrome (Reitmair *et al.,* 1996).

To examine any possible synergism between *Msh2* and *p53* in neoplasia, Cranston *et al.,* (1997) generated ($Msh2^{-/-}$, $p53^{-/-}$) mice. Combined deficiency of *Msh2* and *p53* resulted in developmental arrest of all female embryos at 9.5 days (Cranston *et al.,* 1997). In contrast, male ($Msh2^{-/-}$, $p53^{-/-}$) mice were viable but succumbed to lymphoma more rapidly than either $Msh2^{-/-}$ or $p53^{-/-}$ alone, demonstrating co-operativity between the two genes. Such co-operativity did not appear to arise as a consequence of increased MSI, as tumours arising in ($Msh2^{-/-}$, $p53^{-/-}$) mice did not show significantly increased levels of MSI compared to tumours from $Msh2^{-/-}$ mice. Mice bearing mutant *p53* and *Msh2* alleles mice were intercrossed, and confirm here the phenotype of ($Msh2^{-/-}$, $p53^{-/-}$) mice shown by Cranston *et al.,* (1997). The effect of heterozygosity at each locus was additionally characterised with respect to murine survival and tumour development. Furthermore, the influence of genotype upon the pattern of genetic instability within tumours was investigated. Taken together, these studies demonstrate a novel dose sensitive role for *p53* in suppressing MSI.

6.1 Results

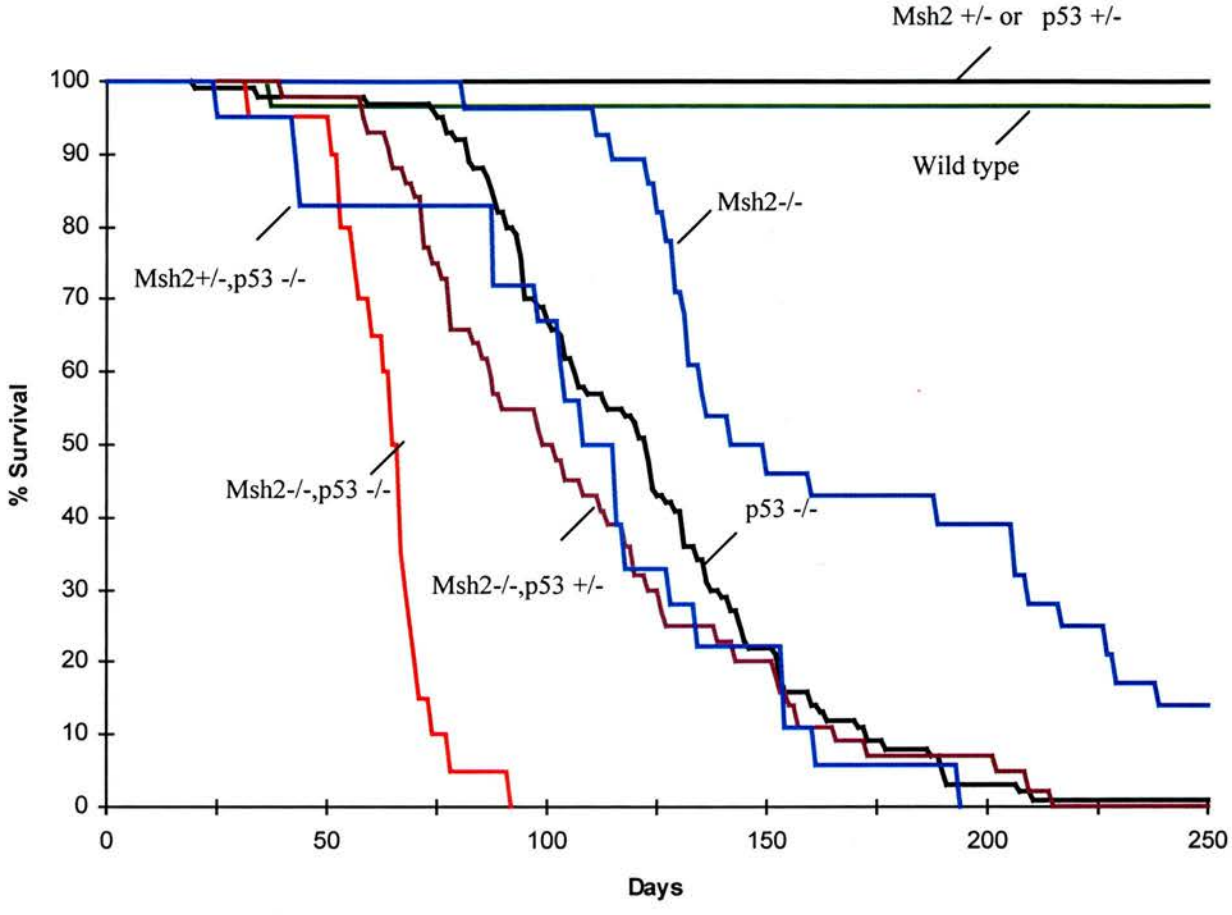
6.1.1 Survival cohort of mice segregating for *Msh2*$^{-/-}$ and *p53*$^{-/-}$

Msh2$^{-/-}$ mice (De Wind *et al.,* 1995) were crossed to *p53*$^{-/-}$ mice (Clarke *et al.,* 1993) to generate a cohort of animals segregating for mutant alleles. This cross was initiated by Neil Toft. Mice with the following genotypes were studied: (*Msh2*$^{-/-}$, *p53*$^{+/+}$), (*Msh2*$^{+/+}$, *p53*$^{-/-}$), (*Msh2*$^{-/-}$, *p53*$^{+/-}$), (*Msh2*$^{+/}$, *p53*$^{-/-}$) and (*Msh2*$^{-/-}$, *p53*$^{-/-}$). Viable animals were obtained for each genotype, and both males and females in all five genotype groups were fertile beyond six weeks of age. In contrast to the findings of Cranston *et al.,* (1997) we did not observe increased rates of female embryonic lethality in (*Msh2*$^{-/-}$, *p53*$^{-/-}$) mice (Toft *et al.,* 1998).

Cohorts of mice were studied to investigate survival rates and tumourigenesis. Colonies were monitored for a period of 240 days, and mice were killed when they appeared visibly ill by at first Neil Toft and then myself. Kaplan-Meier survival plots were generated for all genotypes (Fig. 1). Survival data for *Msh2*$^{+/+}$ *p53*$^{+/-}$ mice was generated from historical controls (Purdie *et al.,* 1994). (*Msh2*$^{-/-}$, *p53*$^{-/-}$) mice became ill significantly earlier than either *Msh2*$^{-/-}$ or *p53*$^{-/-}$ mice alone ($P<$ 0.0006, log rank test). Heterozygosity for *Msh2* on a *p53*$^{-/-}$ background did not significantly alter survival from *p53* deficiency alone ($P>0.09$, log rank test). In contrast, *Msh2* deficiency on a *p53* heterozygote background reduced survival compared with both (*Msh2*$^{+/+}$, *p53*$^{+/-}$) mice ($P<0.00001$, log rank test) and (*Msh2*$^{-/-}$, *p53*$^{+/+}$) mice ($P<0.00001$ log rank test).

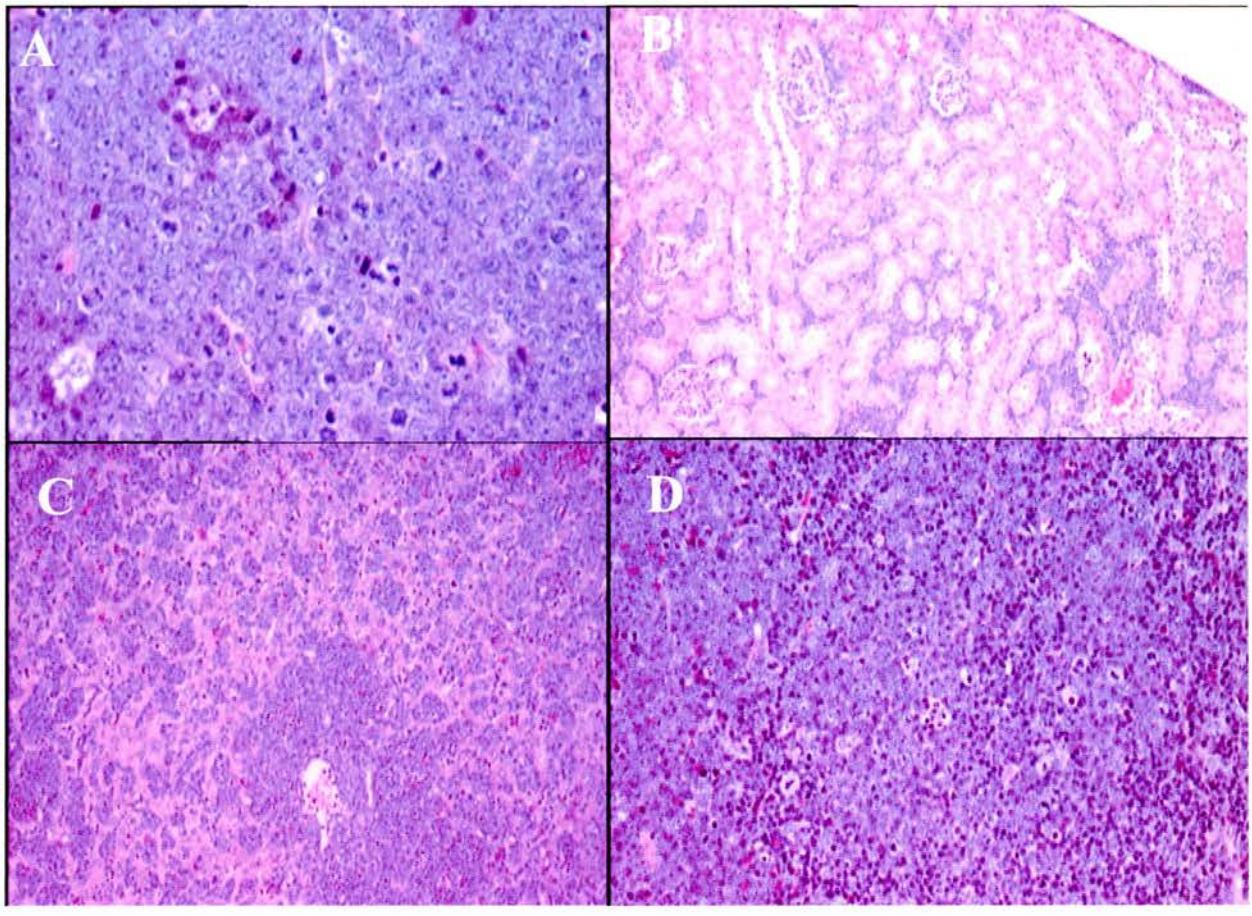
Figure 6.1.1: Kaplan Meier plot of survival

Age of animals is given in days. Survival of $p53^{+/-}$ mice was reproduced from historical data (Purdie *et al.*, 1994). All cohorts of mice are labelled accordingly. Cohort sizes were as follows: ($Msh2^{-/-}$, $p53^{-/-}$) n= 20; ($Msh2^{+/-}$, $p53^{-/-}$) n=18; ($Msh2^{-/-}$, $p53^{+/-}$) n= 47; $Msh2^{-/-}$ n= 28; $p53^{-/-}$ n= 57.



Histological examination showed that all $Msh2^{-/-}$ mice were characterised by lymphoma, with 20% additionally developing intestinal neoplasia. 88% of $p53^{-/-}$ mice developed lymphoma and 12% developed poorly differentiated sarcomas. The tumour spectrum of ($Msh2^{-/-}$ $p53^{+/-}$) mice paralleled that of $Msh2^{-/-}$ mice (95% lymphoma, 11% intestinal neoplasia). The tumour spectrum of ($Msh2^{+/-}$ $p53^{-/-}$) mice paralleled that of $p53^{-/-}$ mice (83% Lymphoma, 22% sarcoma), although 2 of the 4 sarcomas occurring in the ($Msh2^{+/-}$ $p53^{-/-}$) mice were identifiable as leiomyosarcomas of the caecal wall, a tumour type not observed in the $p53^{-/-}$ cohort.

Figure 6.1.2 Tumour histology. Panels A-D display different types of lymphoma from the mice. Over 90% of the mice from all the cohorts died from lymphomas. Panel A shows a thymic lymphoma (x 400), the predominant form of lymphoma. Panel B shows lymphoma infiltration of the kidney (black cells)(x100). Panel C shows lymphoma infiltration of liver (x100). Panel D displays splenomegally (lymphoma of the spleen)(x400).



6.1.2 DNA content of tumours

Neil Toft analysed the DNA content of tumour cells by flow cytometry (Table 6.2.1). The DNA content was classified as aneuploid if an additional G_1 or G_2 peak with a different DNA index was present. Lymphomas from *Msh2*$^{-/-}$, (*Msh2*$^{-/-}$ *p53*$^{+/-}$) and (*Msh2*$^{-/-}$ *p53*$^{-/-}$) mice all displayed a diploid karyotype. In contrast, 2 out of 7 (29%) of the lymphomas arising in *p53*$^{-/-}$ mice showed evidence of aneuploidy. Flow cytometric analysis permits a relatively crude assessment of chromosomal instability.

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Table 6.2.1 Analysis of thymic lymphomas in MSH2 / p53 intercrossed mice by flow cytometry
Flow cytometric analyses of thymic lymphomas from *MSH2*$^{-/-}$ *p53*$^{+/+}$, *MSH2*$^{+/+}$ *p53*$^{-/-}$, *MSH2*$^{-/-}$ *p53*$^{+/-}$, *MSH2*$^{-/-}$ *p53*$^{-/-}$ mice. Tumours were classified as either diploid or as aneuploid. Aneuploidy was identified by the presence of an additional G_1 or G_2 peak within the cellular DNA content.

Thymic Lymphoma	Total number of tumours	Diploid	Aneuploid
MSH2$^{-/-}$	7	7	0
P53$^{-/-}$	7	5	2
MSH2$^{-/-}$, *p53*$^{+/-}$	7	7	0
MSH2$^{-/-}$, *p53*$^{-/-}$	7	7	0

Therefore Lucy Curtis analysed stability using Comparative Genomic Hybridisation (CGH). CGH was performed on a minimum of four to eight metaphase spreads per tumour and an average green/red profile was generated for each chromosome. A green/red ratio outside the limits of 1.2 or 0.8 was scored as an increase or decrease in DNA content for a single chromosome. Tumours arising in *Msh2*$^{-/-}$, (*Msh2*$^{-/-}$ *p53*$^{+/-}$), (*Msh2*$^{+/-}$ *p53*$^{-/-}$) and, perhaps most significantly, (*Msh2*$^{-/-}$ *p53*$^{-/-}$) mice displayed stable genomes with few chromosomal gains or losses identified (Table 6.2.2). In contrast, tumours arising in *p53*$^{-/-}$ mice showed a number of amplified or deleted chromosomal regions

Table 6.2.2 Analysis of thymic lymphomas in MSH2 / p53 intercrossed mice by CGH

Comparative genomic hybridisation (CGH) analyses of murine thymic lymphomas arising in mice of varying *MSH2* and *p53* genotypes. CGH was performed on a minimum of five metaphase spreads per tumour and an average green / red profile generated for each of the 19 mouse autosomes. Tumours were scored as aneuploid if one or more chromosomes displayed a green / red ratio outside of 1.2 or 0.8.

Thymic Lymphoma	Total number of tumours	Diploid	Aneuploid
$MSH2^{-/-}$	6	5	1
$P53^{-/-}$	10	4	6
$MSH2^{-/-}$,$p53^{+/-}$	13	12	1
$MSH2^{+/-}$, $p53^{-/-}$	6	5	1
$MSH2^{-/-}$, $p53^{-/-}$	7	6	1

6.1.3 Microsatellite instability in tumours

To investigate whether the accelerated development of tumours in ($Msh2^{-/-}$, $p53^{-/-}$) mice was associated with a *p53*-dependent increase in MSI, tumours were assessed for instability at four separate microsatellite loci. Tumour and tail were harvested by myself and DNA made and sent to Lucy Curtis for MSI analysis. Data is summarised in table 6.3.1. Compared to tumours arising in $Msh2^{-/-}$ mice, MSI was more frequently observed in tumours arising in ($Msh2^{-/-}$, $p53^{+/-}$) mice (P=0.008 Fisher exact test). In the smaller cohort of ($Msh2^{-/-}$, $p53^{-/-}$) tumours analysed a similar trend was observed, but this difference was not significant (P=0.38 Fisher exact test). These findings indicate that heterozygosity for *p53* increases genomic instability at the nucleotide level on an *Msh2* null background.

Table 6.3.1:MSI in thymic lymphomas from MSH2 / p53 intercrossed mice

Replication Error (RER) status of thymic lymphomas arising in *MSH2 / p53* mutant mice. MSI was determined by analysing four different loci and determining whether the loci were stable or unstable. Tumours with one or more unstable loci were deemed to show MSI and hence RER positivity.

Genotype	Total number of tumours	Microsatellite instability	% RER+ve
$MSH2^{-/-}$	16	8	50%
$p53^{-/-}$	3	0	0%
$MSH2^{-/-}$ $p53^{+/-}$	11	11	100%
$MSH2^{+/-}$ $p53^{-/-}$	7	0	0%
$MSH2^{-/-}$ $p53^{-/-}$	8	7	87.5%

In the above study an increase in MSI was not demonstrated in the complete absence of *p53* (i.e. in tumours arising on a ($Msh2^{-/-}$, $p53^{-/-}$) background), possibly because the sample size analysed was too small. Therefore the analysis was extended to primary fibroblast clones representative of each genotype combination. Primary fibroblast cultures were derived from E14 embryos and cells plated at cloning density by myself. Two weeks later clones were picked and instability scored as before in each clone by Lucy Curtis (table 6.3.2). As predicted, the level of instability was low in wild type clones and higher in *Msh2* null clones. In agreement with the analysis of the tumour samples, heterozygosity for *p53* significantly increased the level of instability (P=0.0005, Yates' corrected chi-squared test). Remarkably, however although the same trend was observed in the double null samples, the increase in the level of instability was only significant at the 10% level .

Figure 6.3.2 Geisma stained fibroblasts clones. After staining clones were picked and DNA made from them (which can be seen as scratches in the clones).

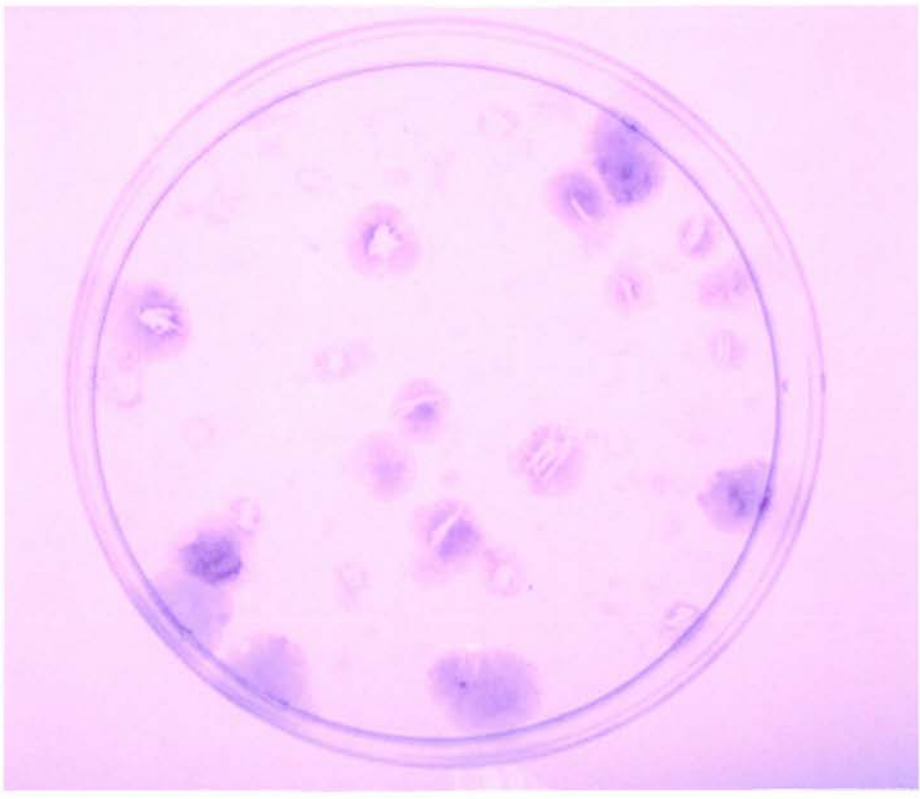


Table 6.3.3.MSI in embryonic fibroblast clones from MSH2/p53 intercrossed mice.

Genotype	Number of shifted clones - 3 sites	Number of shifted clones - 4 sites
Msh2$^{-/-}$ *p53*$^{+/+}$	11/88 (12.5%)	14/86 (16.3%)
Msh2$^{-/-}$ *p53*$^{-/-}$	14/57 (24.6%)	14/47 (29.8%)
Msh2$^{-/-}$ *p53*$^{+/-}$	15/45 (33%)	15/31 (48.4%)

Statistics-Yates' corrected chi-square

	3 sites	4 sites
Msh2$^{-/-}$ vs *Msh2*$^{-/-}$ *p53*$^{-/-}$	*P*=0.0983	*P*=0.1086
Msh2$^{-/-}$ vs *Msh2*$^{-/-}$ *p53*$^{+/-}$	*P*=0.0084*	*P*=0.0005*
Msh2$^{-/-}$ *p53*$^{-/-}$ vs *Msh2*$^{-/-}$ *p53*$^{+/-}$	*P*=0.45	*P*=0.1544

MSI in primary cultures derived from mice of varying *MSH2* and *p53* genotypes. MSI was assessed at either 3 loci (D7Mit17, D10Mit2 and D14Mit15) or at 4 loci (D1Mit4 in addition).

6.1.4 *p53* status of tumours from (*Msh2*$^{-/-}$, *p53*$^{+/-}$) mice and clones

These results raised the possibility that heterozygosity for *p53* was sufficient to increase the levels of microsatellite instability, and indeed that a reduction in *p53* levels has a more dramatic affect than the complete absence of *p53*. This was of particular interest as heterozygosity for *p53* has been demonstrated to predispose to malignancy (Venkatachalam *et al.,* 1998). Therefore the status of the wild-type *p53* allele in tumours arising in (*Msh2*$^{-/-}$, *p53*$^{+/-}$) mice was assessed. Loss of heterozygosity was not observed in any of the eighteen thymic lymphomas examined by PCR, demonstrating that gross chromosomal deletion of the remaining wild-type *p53* allele had not occurred. Three tumours were analysed by sequencing by Neil Toft and a single G→A transition was observed at codon 234 in one of these tumours, predicted to give an amino acid change from methionine to isoleucine. This suggested that a percentage of tumours may have retained functional *p53*. To determine if this were the case *p53* activity was assessed by analysing the levels of p53 and p21 by Western analysis six hours following exposure to 5 Gy gamma-irradiation (figures 6.4.1 and 6.4.2). In wild type tissue p53 became stabilised and p21 levels were increased. No p53 was detectable in tumours arising on a *p53* null background, nor was induction of p21 observed. However, induction of p21 was observed in 4 out of 18 tumours arising in mice heterozygous for *p53*, all of which showed stabilisation of p53. One further tumour showed stabilisation of p53 but no transactivation of p21, suggesting mutation of *p53* consistent with our sequencing data. To confirm that this reflected retention of *p53* activity we performed Electrophoretic Mobility Shift Assay (EMSA) analysis upon the same material, and established that *p53* specific DNA binding does occur within tumours arising on a heterozygous background (figure 3B). Taken together, these results strongly argue for retained *p53* function in a significant subset of tumours arising on an *Msh2* null, *p53* heterozygous background.

Similar approaches were used to determine if *p53* function had been retained in the primary fibroblast cultures obtained from *p53* heterozygous, *Msh2* mutant embryos. I used immunohistochemical and Western analysis to determine *p53* functionality in mass cultures of primary fibroblasts. These were maintained at low density for two weeks prior to harvest such that they had undergone a similar number of divisions to those cells analysed as single clones for MSI. Six hours following exposure to DNA damage (10J UVB irradiation for immunohistochemistry, 5 Gy for Western analysis) *p53* null cultures did not show upregulation of p21. In contrast, wild type

and *p53* heterozygous cultures showed p53 stabilisation (figure 6.4.1) and upregulation of p21 (figure 6.4.2). Furthermore, individual immunohistochemical analysis of individual heterozygous clones showed that *p53* was upregulated following DNA damage in $p53^{+/-}$, $Msh2^{-/-}$ cultures (figure 6.4.1A). Taken together these data argue very strongly that *p53* functionality was retained in *p53* heterozygous cells over the period of cloning, and therefore that the increase in microsattelite instability reported above occurs in a functional *p53* heterozygous background.

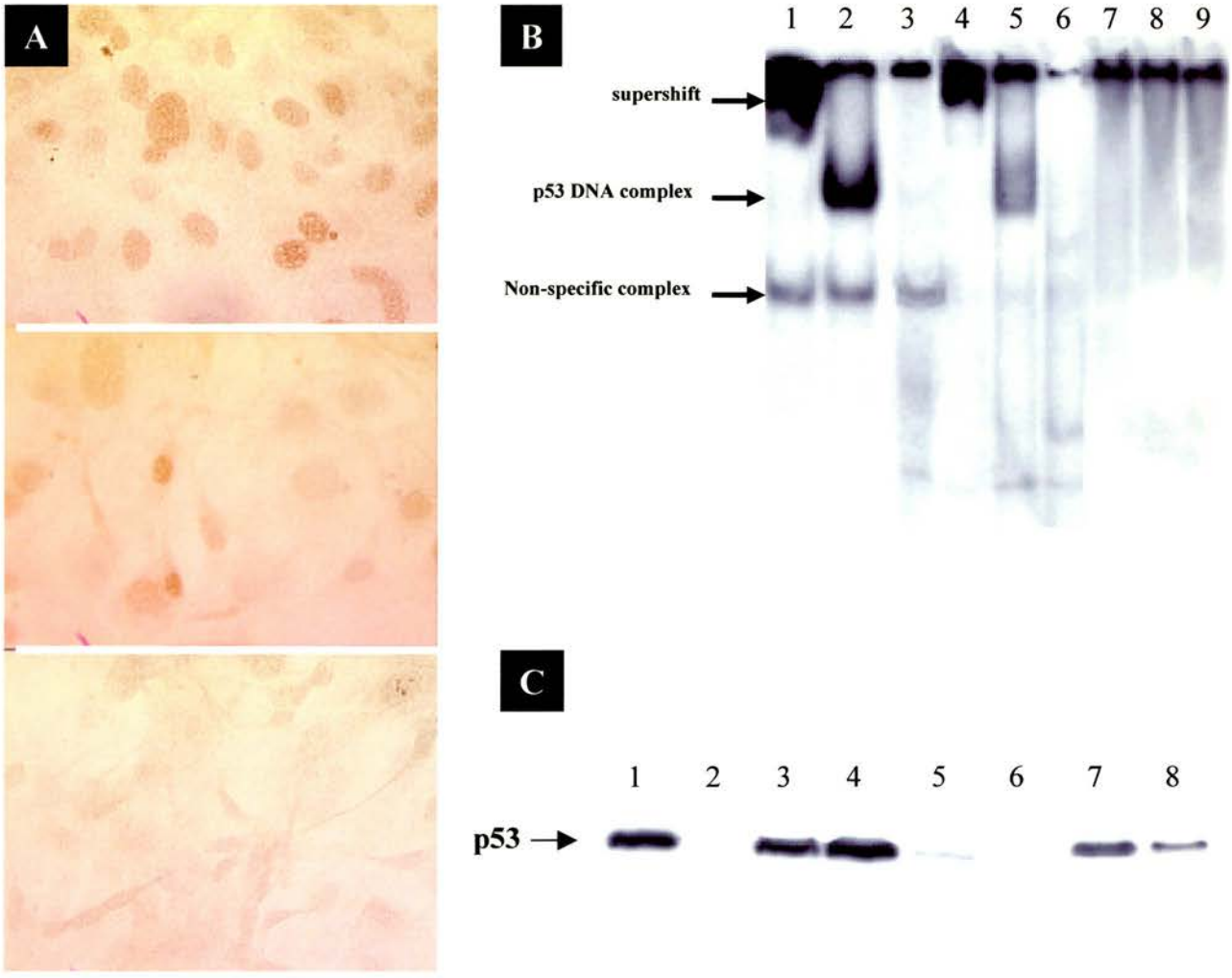


Figure 6.4.1
Analysis of *p53* status
A) *p53* immunohistochemistry of fibroblast clones. Top panel, *MSH2-/- p53+/-* clones 6 hours following 10J UV irradiation. Middle panel, mock treated *MSH2-/- p53+/-* fibroblast clones. Bottom panel, *Msh2-/- p53-/-+/-* clones 6 hours following 10J UV irradiation.

B) EMSA analysis of *p53* DNA binding activity in a tumour sample which showed *p53* stabilisation and p21 induction following DNA damage. Lanes 1-3, wild type ES cells following 10J UV damage. Lanes 4-6, *Msh2-/- p53+/-* tumour sample following 5Gy gamma irradiation. Lanes 7-9, *p53* null tumour following 5Gy gamma irradiation. Lanes 1,2,4,5,7 and 8 reflect incubation with a *p53* specific DNA oligonucleotide which resulted in *p53*-DNA complex formation in lanes 2 and 5. Lanes 3, 6 and 9 reflect incubation with a DNA oligonucleotide to which *p53* does not bind. Lanes 1,4,7 reflect additional incubation with a *p53* antibody which resulted in supershifts in lanes 1 and 4.

C) *p53* Western Analysis 6 hours following 5Gy gamma irradiation. Lanes 1,3,4 and 5, irradiated (*Msh2-/-,p53+/-*) tumours; lane 2, mock treated (*Msh2-/-,p53+/-*) tumour; lane 6 irradiated *p53-/-* tumour. Lane 7, irradiated (*Msh2-/- p53+/-*) pooled fibroblast clones. Lane 8, mock treated pooled fibroblast clones. In total 5 tumours showed *p53* stabilisation following DNA damage, 3 of which are shown in this figure (lanes 1,3,and 4).

Figure 6.4.2

Analysis of p21 status. Western Analysis 6 hours following irradiation with 5Gy gamma irradiation. Top panel; lanes 1 and 3-8 irradiated *Msh2-/- p53+/-* tumours. Lane 9, mock treated *Msh2-/- p53+/-* tumour. Lane 2, irradiated *Msh2-/- p53-/-* tumour. Bottom panel. Lane 1, mock treated pooled *Msh2-/- p53+/-* fibroblast clones. Lanes 2-3, pooled *Msh2-/- p53+/-* irradiated fibroblast clones. Lanes 4-5, irradiated spleen and thymus from a wild type mouse. Lane 6, irradiated *p53* null tumour. Lanes 7, irradiated spleen and tumour from a *Msh2-/- p53+/-* mouse. Lane 8, irradiated tumour from a *Msh2-/- p53+/-* mouse. Lane 9 is a positive control obtained from Kim2 cells overexpressing p21.

6.2 Discussion

Cranston *et al.,* (1997) have previously described the phenotype of mice functionally null for both *p53* and *Msh2.* They found that females died in utero, but that males developed normally to birth, although they subsequently succumbed to lymphoma earlier than either *p53* or *Msh2* singly null mice (Cranston *et al.,* 1997). A similar intercross was generated here, using different null alleles (Clarke *et al.,* 1993, De Wind *et al.,* 1995). As have been previously reported, there was no increased rates of female embryonic lethality in ($Msh2^{-/-}$, $p53^{-/-}$) mice in these strains (Toft *et al.,* 1998). This difference probably arises from differences in genetic background, as has recently been strongly argued by Cranston and Fishel (1999). Data summarising survival and tumour spectrum in all the genotype groups agree well with previously published work (De Wind *et al.,* 1995; Reitmair *et al.,* 1995 and 1996a; Purdie *et al.,* 1994; Cranston *et al.,* 1997). This suggests that no fundamental differences exist between the targeted alleles used in this study and those used by Cranston *et al.,* (1997).

6.2.1 Accelerated tumourigenesis in ($Msh2^{-/-}$, $p53^{+/-}$) mice:

Heterozygosity for *p53* reduced survival on an $Msh2^{-/-}$ background, the most obvious mechanism for this is through inactivation of the remaining wild type *p53* allele. However there was no evidence for this by PCR and furthermore sequencing of the 'mutation hotspot' (Vogelstein and Kinzler, 1992) failed to identify mutations in 2 out of 3 tumours. These observations raised the possibility that *p53* function had been retained in at least a subset of tumours as has previously been reported (Venkatachalam *et al.,* 1998) and therefore it was tested if *p21* could still be transcriptionally activated following DNA damage. This is a particularly potent test of *p53* activity as upregulation of p21 in these circumstances is absolutely dependent upon *p53* and because it has recently been confirmed that the transcriptional activity of *p53* is essential for many *p53*-dependent process including apoptosis, cell cycle arrest and tumour suppression (Jimenez *et al.,* 2000, Choa *et al.,* 2000). Western analysis of both p53 and p21 within the samples confirmed retention of *p53* activity in a proportion (25%) of tumours, demonstrating that a reduction in *p53* gene dosage can be sufficient to accelerate tumourigenesis.

6.2.2 Tumourigenesis is not accelerated in (*Msh2*$^{+/-}$, *p53*$^{-/-}$) mice

Heterozygosity for *Msh2* in a *p53* null environment did not accelerate spontaneous tumourigenesis. This is perhaps not surprising since *Msh2*$^{+/-}$ cells do not show MSI (De Wind *et al.,* 1995; Kolodner, 1995). However, this finding also suggests that the remaining wild-type *Msh2* allele does not act as a target for mutation even though *p53* deficiency has been shown to cause genomic instability (Bouffler *et al.,* 1995) and DNA amplification (Yin *et al.,* 1992). These observations can perhaps be reconciled by the fact that *p53* deficiency does not necessarily lead to an increase in the *in vivo* spontaneous mutation frequency (Clarke *et al.,* 1997). In chapter 7, it will be shown that *Msh2* heterozygotes have increased mutation at old age. As there is no increase in tumourigenesis in the (*Msh2*$^{-/-}$, *p53*$^{-/-}$) mice, this again argues that p53 deficiency is driving tumourigenesis in a MMR-independent manner in the (*Msh2*$^{-/-}$, *p53*$^{-/-}$) mice. Heterozygosity for *Msh2* did weakly influence the site of sarcoma development. This must reflect complex tissue specific gene dependency in the prevention of neoplasia, a feature previously reported in several different intercrosses (e.g. Clarke *et al.,* 1995, Sansom and Clarke 2000).

Taken together, the survival studies indicate that heterozygosity for *p53* has a profound effect upon *Msh2* dependent tumorigenesis, but that the inverse is not true. This directly suggests that *p53* may be involved in modulation the levels of MSI within an MMR deficient background; a hypothesis that was tested by analysing levels of MSI in tumour samples.

6.2.3 Increased MSI conferred by heterozygosity for *p53*

Colorectal cancers exhibit two distinct forms of genomic instability, which have been classified by Lengaurter *et al.,* (1997) as: MIN (microsatelitte instability, MSI), predicted to be associated with mutations in the MMR genes; and CIN (chromosomal instability), predicted to associated with loss of *p53* and the development of aneuploidy. The data presented here directly contradicts this view as it is demonstrated that, in the absence of *Msh2*, *p53* can function to suppress MSI and that a reduction in *p53* gene dosage decreases tumour latency specifically through increasing MSI.

Suppression of MSI in the absence of *Msh2* appears to be a general function of *p53*, as it occurs not only within tumour derived samples, but also within primary fibroblasts derived from the mutant strains. The results apparently contradict previous data obtained by Cranston *et al.,* (1997).

However, their study did not include mice heterozygous for *p53*, for which the clearest phenotype was observed. Furthermore, where both studies compared *Msh2*$^{-/-}$ and (*Msh2*$^{-/-}$ *p53*$^{-/-}$) mice a similar trend to increased instability was observed in the absence of *p53*, although in neither study did this attain significance at the 5% level.

Two possible explanations may account for the *p53* dependent increase in MSI. First, deficiency of *p53* may reduce genomic instability at the nucleotide level by mediating repair, either directly or indirectly. This is supported by the observation that *p53* is capable of binding insertion/deletion loops, the DNA lesion associated with MSI (Lee *et al.,* 1995). A second possibility is that *p53* may normally function by clearing (through apoptosis) cells characterised by instability. Both *p53* and *Msh2* have well-characterised pro-apoptotic roles following damage of mismatch type (Clarke *et al.,* 1993; Lowe *et al.,* 1993, Toft *et al.,* 1999, Sansom and Clarke 2000), and again the ability of *p53* to bind insertion/deletion loops supports a direct role for *p53* in lesion recognition.

Currently it is not possible to discriminate between these possibilities, but it is clear from these results that *p53* status only becomes relevant to MSI in the absence of *Msh2*, as singly mutant *p53* deficient mice are not characterised by increased MSI. This strongly suggests that *p53* is redundant to the normal monitoring and clearance of MSI associated lesions, but that following loss of *Msh2* the presence of normal levels of *p53* becomes essential to this process.

These findings characterise an interaction between *Msh2* and *p53* mutations in increasing MSI and tumour development. Remarkably the largest increase in MSI occurs in p53 heterozygotes, suggesting that complete ablation of *p53* leads to the upregulation of relatively efficient compensatory mechanisms, perhaps mediated by one of the growing family of *p53* homologues. In human colorectal cancer coincident mutations in both *Msh2* and *p53* have rarely been reported (Cottu *et al.,* 1996), although examples of combined microsatellite and chromosomal instability do exist (Lengauer *et al.,* 1997). One simple explanation for the absence of combined mutation is that retained heterozygosity for *p53* is difficult to establish and is not routinely screened for. Indeed, the results presented above do not predict combined microsatellite and chromosomal instability, as double mutants are characterised by enhanced MSI and diminished aneuploidy.

In conclusion, these results demonstrate a novel pathway to tumorigenesis by establishing a role for *p53* in maintaining integrity at the nucleotide level and they also raise the strong possibility that

combined mutation of both *p53* and the DNA mismatch repair proteins are currently under reported in human colorectal tumourigenesis.

Chapter 7: *Msh-2* suppresses *in vivo* mutation in a gene dose and lesion dependent manner.

7.0 Introduction

The majority of HNPCC patients are germline heterozygotes for either *MLH1* or *MSH2*. Thus far this thesis has examined the phenotypes of mice homozygous for these mutations. Therefore this chapter investigates whether heterozygosity for *Msh2* affects gene dependent apoptosis or mutation accumulation.

Mice heterozygous for *Msh2* and *Mlh1* do not show reduced survival compared to wild type controls, nor are they predisposed to increased intestinal neoplasia (De Wind *et al.* 1998, and chapter 6). Heterozygosity for *Msh2* (unlike homozygosity) also did not accelerate intestinal neoplasia on an $Apc^{Min/+}$ background (De Wind *et al.*, 1998). Mice heterozygous for *Msh2* are however characterised by increased tumorigenesis. Surprisingly when these tumours were examined for Loss of Heterozygosity (LOH) only 1 out of 71 had lost the remaining copy of *Msh2* and this was the only tumour to exhibit microsatellite instability (MI), indicating that the majority of tumours truly were hemizygous for *Msh2* (De Wind *et al.*, 1998). These results are therefore consistent with a hemizygous effect of *Msh2*.

In this thesis two possible biological mechanisms underlying the relationship between MMR deficiency and neoplasia have frequently been discussed. First, that failed recognition and repair of mismatch lesions leads directly to an increase in mutation frequency and thereby to malignancy. Second that failure to engage MMR dependent apoptosis may also predispose to malignancy. Consistent with this second theory is that it has been shown (both in vitro and in vivo) that *Msh2* deficiency is associated with increased long term survival following alkylating agents (Toft *et al.*, 1999, chapter 5). In addition *Msh2* deficiency leads to an elevation in mutation frequencies following exposure to DNA damage (Andrew *et al.*, 1998; Toft *et al.*, 1999). However in chapter 5, the failure to engage an apoptotic response was shown to be a poor predictor of survival. Likewise, the precise relationship between the ability to engage apoptosis and mutation surveillance remains relatively poorly defined.

To investigate the *in vivo* consequences of hemizygosity at the *Msh2* locus and to further explore the relationship between apoptosis and mutation surveillance the gene dependency of these two endpoints have been examined in the small intestine of mice

wild type, heterozygous and nullizygous for *Msh2*. These studies were performed both at spontaneous levels of damage and following exposure to the DNA damaging agents MNNG and cisplatin.

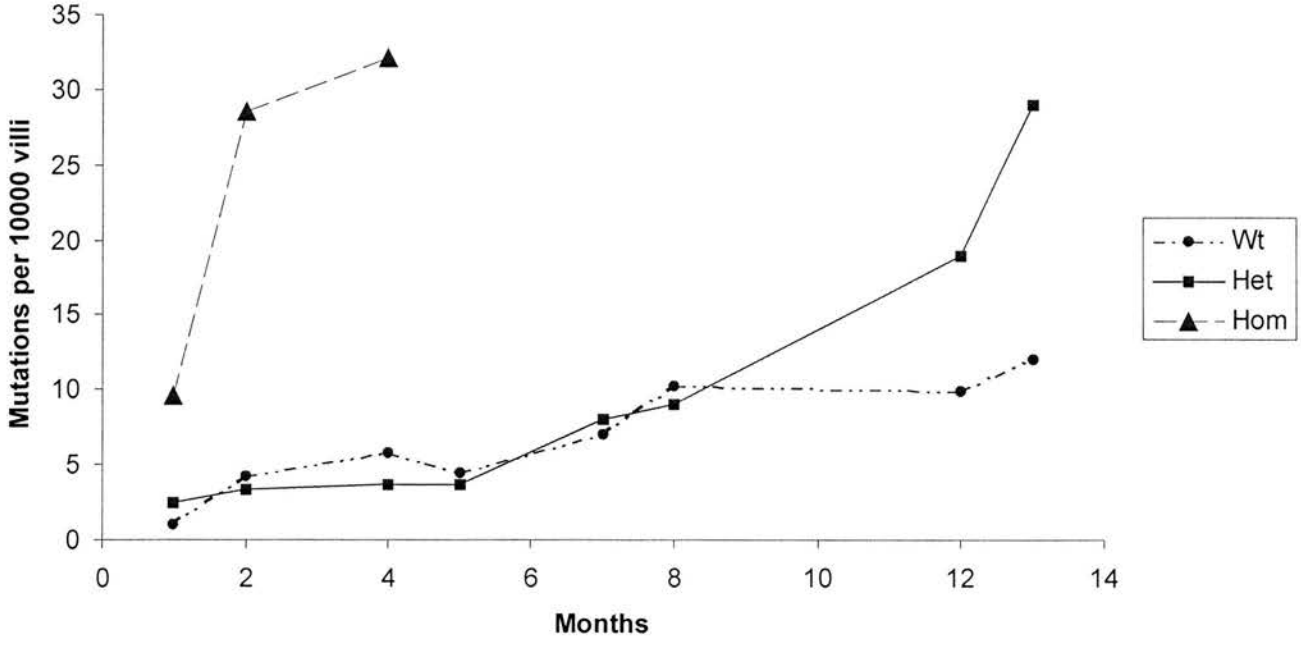
7.1 Results

7.1.1 Mutation frequency is increased in Msh2 heterozygous mice over 1 year old

Mutation frequency was scored at the *Dlb-b1* locus at spontaneous levels of DNA damage over a 13 month time course (figure 7.2.1). *Msh2*$^{-/-}$ mice exhibited a higher mutation frequency than wild type controls and *Msh2* hemizygous mice. *Msh2*$^{-/-}$l mice showed a marked increase in mutation frequency from 4 to 8 weeks: a period where there is a significant increase in the number of crypts (Shoemaker *et al.,* 1995). However after this period there was not a significant increase in the mutation frequency in the *Msh2*$^{-/-}$ mice.

The most intriguing result was that there was a significant increase in mutation frequency in the heterozygotes aged over 12 months (n=10) compared to wild types (n=10) (p=0.04, Mann Whitney).

Figure 7.1. Increased mutation frequency in the aged Msh2 heterozygotes. Mutation frequency per 10000 villi was scored at the dlb-1b. At least 3 mice were used for each timepoint. Dashed line with circular points, wild type mice; solid line with square points, *Msh2*$^{+/-}$ mice; Dashed line with triangular points, *Msh2*$^{-/-}$ mice.



7.1.2 Intact apoptotic response in the heterozygotes

The above observations prompted analysis of the apoptotic response, to determine if this response was also *Msh2* gene dose sensitive. Apoptosis of enterocytes within the crypts of Lieberkuhn was examined at 6 hours after drug exposure (figure 7.2.)

Figure 7.2: The *in vivo* apoptotic response at 6 hours following drug exposure. For each animal 50 half crypts were analysed; the total number of apoptotic bodies scored in all crypts analysed is plotted on the vertical axis. A) Following 50mg/kg of MNNG, B) Following 10mg/kg of cisplatin. Closed bars, wild type mice; grey bars, hemizygous mice and open bars, *Msh2* null mice. Columns represent mean, errors bar represents SD. At least 3 mice were used for each point.

Figure 7.2A. MNNG

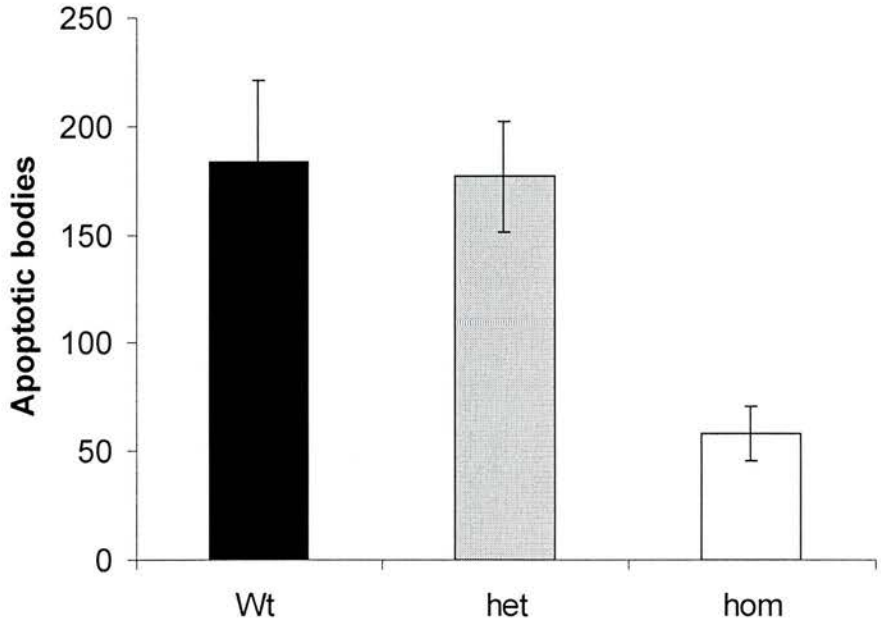
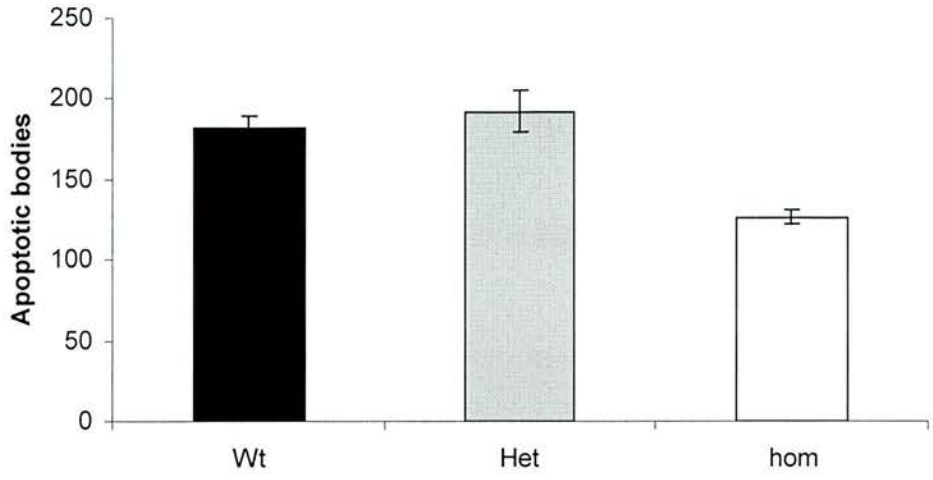


Figure 7.2B Cisplatin



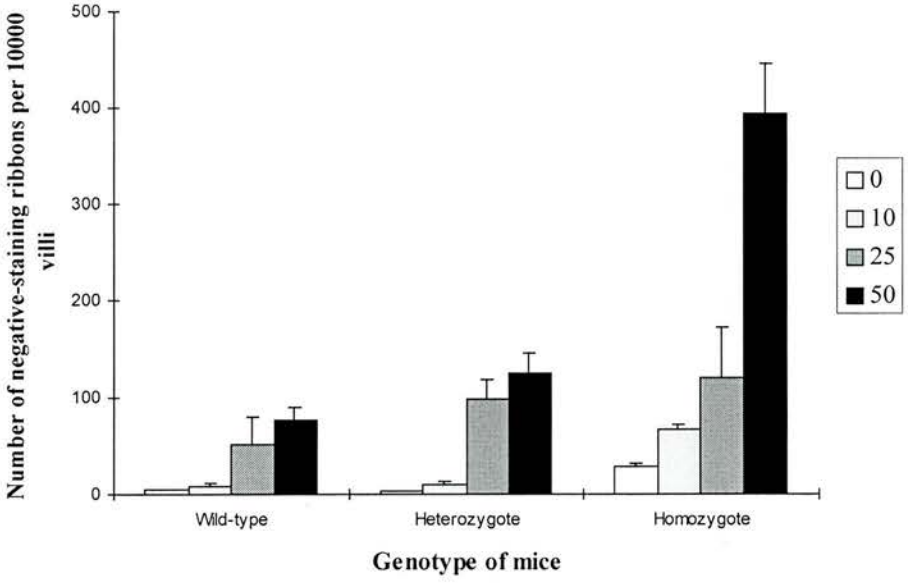
It had previously been established that this time point coincides with the peak induction of apoptosis within this structure (see Toft *et al.,* 1999, Sansom and Clarke 2000). Consistent with previous results *Msh2*$^{-/-}$ deficient mice showed a reduced apoptotic response following exposure to MNNG whilst there was a normal apoptotic response in wild type and *Msh2*$^{+/-}$ mice (p=0.38). Following exposure to cisplatin, no difference was observed between hemizygotes and wild type mice (p=0.20, Mann Whitney)

7.1.3 No increase in induced mutation frequency in Msh2 heterozygous mice

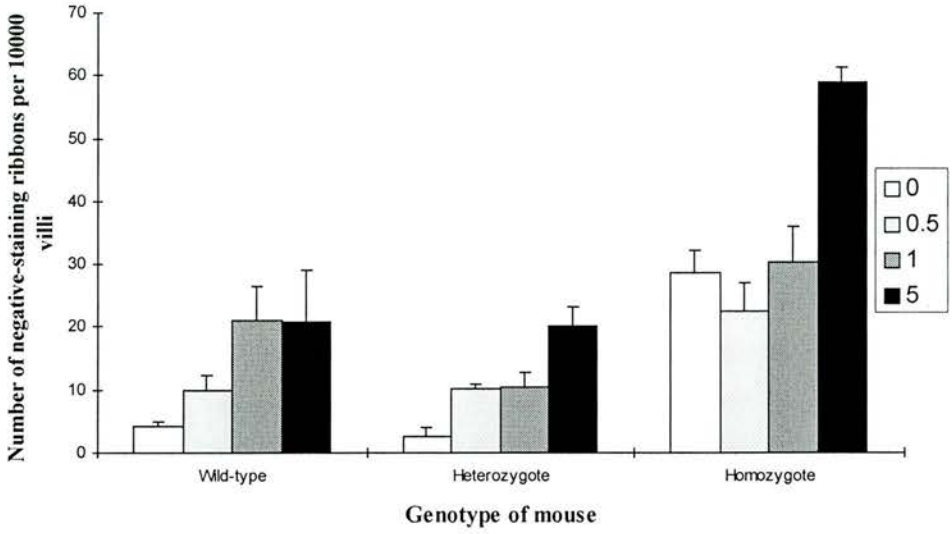
Despite the presence of a normal, intact apoptotic response in *Msh2* heterozygotes it remained possible that there could be a gene dosage effect in mutation induction following these agents. Previously Shcherbakova and Kunkel (1999) have demonstrated significant increases in mutation frequency in yeast heterozygous for *Mlh1* following exposure to either bleomycin or UV irradiation. These phenomena were assumed to arise from damage enhanced LOH. Furthermore, studies using *Mlh1* heterozygous mice following NMNU (N-methyl-N-nitrosourea) exposure have revealed increased rates of tumourigenesis compared to wild type controls (Kawate *et al.,* 2000). Therefore mutation frequency was investigated at the *Dlb-1b* locus in young (8-12 weeks old) wild type and *Msh2* mutant mice following treatment with either MNNG or cisplatin.

Figure 7.3: Induced *in vivo* mutation frequency at the *Dlb-1b* locus. Mutation frequencies were determined from intestinal wholemounts 21 days after ip drug injection: Columns represent mean mutation frequency at the *Dlb-1b* locus at the drug dosage shown. At least 3 mice were used per each column. Error bars represent SD.

A) Mutation frequency following MNNG. Open bars, mock treatment; light grey bars, 10mg/kg; dark grey bars, 25mg/kg; closed bars, 50mg/kg. At 50 mg/kg *Msh2* nulls have a significantly higher mutation frequency than wild type and heterozygous mice ($p = 0.04$, Mann Whitney U test)



B Mutation frequency following cisplatin. Open bars, mock treatment, light grey bars, 0.5 mg/kg; dark grey bars, 1 mg/kg; closed bars, 5mg/kg.



Mutation was scored three weeks post intra-peritoneal injection. Following exposure to 50mg/kg MNNG a significant increase in mutation frequency in *Msh2*$^{-/-}$ mice compared to controls was observed (p=0.04, Mann Whitney), but not in heterozygotes (p=0.17, Mann Whitney). These results parallel those obtained following exposure to the alkylating agent Temozolomide (Toft *et al.,* 1999 and chapter 4), confirming that Msh2 normally suppresses mutation following damage of alkylation, but also showing that this response is not *Msh2* gene-dose sensitive.

Following cisplatin exposure *Msh2*$^{-/-}$mice showed increased mutation frequencies relative to controls and heterozygous mice, however these increases could entirely be accounted for by the increase in spontaneous mutation frequency.

7.2 Discussion

7.2.1 Spontaneous Mutation frequency

Previously *Msh2*$^{-/-}$ mice have been shown to have increased mutation frequency at the *Dlb-1b* locus compared to wild type mice (Toft *et al.,* 1999). Here it is shown that there is a very large increase in mutation frequency from 4 to 8 weeks of age which coincides with a period where there is a significant increase in the numbers of crypts in the murine intestine (Shoemaker *et al.,* 1995). Surprisingly, beyond this time point there was no further increase in mutation frequency. This may reflect selection for mice with a reduced predisposition to neoplasia (and potentially reduced mutation frequencies) at the later time points as the peak incidence of neoplasia occurs at 3-4 months.

The most remarkable result was that there was a significant increase in aged *Msh2* heterozygous mice. Similar results have been shown using very sensitive mutation screens in *Msh2* and *Mlh1* heterozygous diploid yeast (Shcherbakova and Kunkel *1999,* Drotschmann *et al.,* 1999,2000). The authors of these studies argued that this increased mutation frequency occurred due to loss of heterozygosity in a small population of the heterozygote cells. However, although LOH at the *Msh2* locus cannot be formally ruled out as the mechanism underlying the increase in the aged hemizygotes, this appears unlikely as this requires two mutations (inactivation of the remaining *Msh2* allele and subsequent inactivation of *Dlb-1b*) to occur sequentially within the same stem cell. Wild type cells accumulate on average 1 additional mutation per 10000 crypts each month (Winton *et al.,* 1988). If it is assumed a null hypothesis and that heterozygosity for *Msh2* plays no direct role in suppressing mutation, and furthermore that *Msh2* is inactivated at a similar rate to *Dlb-1b*, then after 13 months there would be approximately 13 *Msh2* null clones per 10000 crypts. This analysis shows *Msh2* deficiency increases the mutation frequency to, at maximum, less than 10 times that found in wild type cells (the highest fold difference of 9.6 was observed at one month). This indicates that the *additional* mutation burden from the *Msh2* null clones would be less than 1 mutation per 10000 villi. This increase is clearly insufficient to account for the actual observed increase, strongly

implying that the elevation in mutation frequency is occurring as a direct consequence of *Msh2* heterozygosity.

The data presented here parallels other studies which have suggested gene dose sensitivity for *Msh2* dependent responses. These include analyses of the level of DNA lesion and the apoptotic response following exposure to low level radiation treatment (De Weese *et al.,* 1998), and also by studies which have shown very low rates of LOH at the *Msh2* locus in tumours arising in *Msh2* hemizygotes (De wind *et al.,* 1998).

7.2.2 Induced apoptosis and mutation frequency

There are 2 possible explanations for the increase in mutation rate in the heterozygous mice which would have ramifications for the experiments examining the effect of gene dosage on apoptosis and mutation.

First, that there is a subtle defect in repair throughout the lives of the heterozygous mice that only becomes significant when the mice reach old age.

Second, that old heterozygous mice and not young heterozygous mice are defective in repair. If only aged mice are defective in repair, apoptosis and induced mutation should be studied in aged mice as well. Mice are currently being aged to examine this. The most obvious reason for old mice and not young being defective in repair would be that there is loss of the remaining wild type allele. Analysis of the predicted increase in mutation following such LOH argues against this as a mechanism.

As has been shown by Toft *et al.,* (1999) *Msh2* deficiency causes a reduced apoptotic response to alkylation damage. However *Msh2* heterozygosity did not cause a reduction in the apoptotic response.

Msh2 heterozygous mice also had a normal apoptotic response to cisplatin, a result which is perhaps not unexpected as *Msh2*$^{-/-}$ mice show only a marginal reduction in the apoptotic response compared to wild type controls (Toft *et al.,* 1999, chapter 5). The failure to observe high levels of MMR-dependent cell death following cisplatin

exposure contradicts the scenario reported for ovarian cancer cells, where resistance to cisplatin is associated with loss of MMR function and restoration of MMR causes re-sensitisation. This must either be a reflection of the rather subtle changes in the ability to engage apoptosis, or must implicate non-apoptotic MMR-dependent mechanisms in long term cell survival.

Again *Msh2* heterozygosity had no effect on mutation induction following MNNG or cisplatin. *Msh2* deficiency did induce a significant increase following exposure to MNNG. This confirms early studies with the alkylating agents NMNU and temozolomide that *Msh2* in important in recognition of O6 methyl guanine adducts.

However the results fail to demonstrate a role for *Msh2* in mutation surveillance following cisplatin treatment and question the significance of mismatch repair in the clearance of cisplatin-induced DNA damage in normal cells. This failure to observe MMR dependent increase in mutation in response to cisplatin concords with the clonogenic survival data presented in chapter 5 where *Msh2* deficiency (unlike *p53* deficiency) does not cause an increase in long term crypt survival following cisplatin treatment. The findings presented here compliment recent studies by Branch *et al.*, (2000), who demonstrated that loss of MMR was only a minor contributor to cisplatin resistance in ovarian tumour cell lines.

The studies reported here have been performed in normal intestinal enterocytes. It remains possible that the reliance upon functional MMR for mutation surveillance markedly differs between normal and neoplastic cells. Pertinently, Strathdee *et al.*, (2001) have shown that mouse embryonic fibroblasts deficient for *Msh2* still undergo a G2 arrest following cisplatin treatment in marked contrast to MMR deficient tumour cell lines which lose this checkpoint (Brown *et al.*, 1997, Strathdee *et al.*, 2001).

In summary, an extremely complex reliance upon *Msh2* in mutation surveillance has been demonstrated. *Msh2* is critical in monitoring spontaneous levels of DNA damage, such that even a 50% reduction in *Msh2* gene dosage can elevate mutation frequency. This reliance upon *Msh2* in response to spontaneous levels of DNA damage is not directly reflected by gene dependencies in either the apoptotic response or mutation burden following acute DNA damage. *Msh2* deficiency leads to an

increase in the *in vivo* mutation frequency following MNNG treatment, but remarkably this is not the case following exposure to cisplatin. Taken in the context of the recent proliferation of data relating to MMR-dependent DNA damage responses, these observations show that reliance upon functional MMR is highly lesion type dependent. They also challenge our understanding of MMR-dependent suppression of mutation, perhaps partly because MMR dependent responses are usually interpreted in an experimental setting following high, non physiological levels of DNA damage. Clearly, although such scenarios may directly relate to the response to chemotherapeutics in a clinical setting, they may not accurately reflect the reliance upon MMR in normal tissues exposed to low levels of spontaneous damage.

Chapter 8: *Mbd4* is a mediator of the cellular response to DNA damage and suppresses intestinal tumorigenesis.

8.0 Introduction

Mbd4 (*Med1*) was originally identified in a database search for proteins with a methyl binding domain. This search pulled out 4 novel proteins Mbd1, Mbd2, Mbd3 and Mbd4 (Hendrich and Bird 1998). Previously it had been shown that there were 2 methyl binding activities in the cell MeCP1 (Methyl CpG binding protein 1) and MeCP2 (Meehan *et al.*, 1989, Lewis *et al.*, 1992).

As was mentioned in 1.4.6 the majority of CpGs in the genome are methylated as 5 methyl cytosine (appox 85%) apart from large CpG islands in the promoters of genes which are unmethylated. It is that thought methylation is important for tissue specific control of transcription as methylation of promoters causes transcriptional silencing (Tweedie *et al.*, 1997, Razin 1998). The best example of this is the dense methylation of CpG promoters on the inactivated X chromosome and the Xist gene on the active X chromosome (Hendrich and Bird 2000).

Methylation is essential for development and mutations in the enzyme responsible for maintaining methylation DNA (cytosine 5) methyltransferase (DNMT1) results in embryonic lethality. Embryos show a developmental delay at E 9.5 days and then fail to develop past E12.5 (Li *et al.*, 1992). DNMT1 is a maintanance methylase and is not a very efficient *de novo* methylase (Laird 2000). More recently 2 further mammalian methyl transferases have been discovered DNMT3α and DNMT3β again through sequence identity to DNMT1 (Okano *et al.*, 1998). Okano *et al.*, (1999) showed that DNMT3α and DNMT3β were essential for de novo methylation and for normal mouse development.

Methylation also appears to be important for maintaining genomic stability. DNA hypomethylation has been associated with ICF (Immunodeficiency, centromeric instability and facial abnormalities) and more recently mutations in DNMT3β have been found in ICF pateints (Hansen *et al.*, 1999). ES cells nullizygous for

DNMT1 show increased mutation frequencies at both the *Hprt* (hypoxanthine phosphribosyltransferase) and the *tk* (thymidine kinase) locus (Chen *et al.,* 1998). When the spectrum of mutations at these loci were investigated, it was show that gene deletions were caused through mitotic recombination or chromosomal loss accompanied by gene duplications. Other evidence supporting the role of methylation in protecting against genomic instability comes from studies using DNMT1 nullizygous fibroblasts. Using cre-mediated deletion of DNMT1 in fibroblasts, Jackson-Grusby *et al.,* (2001) showed that demethylation of fibroblasts induced apoptosis. This apoptosis was p53 dependent, indicative that apoptosis was induced through some type of DNA damage or genomic instability. Therefore it is quite possible that loss of proteins that are important in binding and maintaining methylation could be important for a number of diseases and cancers.

The methyl binding domain was first characterised from MeCP2 and this was used to search the databases. MeCP2 has 2 functional domains within the protein, the MBD domain and a transcriptional repression domain that recruits histone deacetylase to cause transcriptional silencing. MeCP2 can bind a single CpG pair and is localised to methyl CpG rich heterochromatin regions in mouse cells (Nan *et al.,* 1996). Germline mutations in MeCP2 have been found in patients with Rett Syndrome. This is a neurodevelopmental disorder that affects 1 in 10000 females. *MeCP2* deficient mice also develop a neurological phenotype similar to Rett Syndrome at around 6-10 weeks (Chen *et al.,* 2001, Guy *et al.,* 2001).

Mice have been made that are deficient for the proteins *Mbd2*, *Mbd3* and *Mbd4* (Hendrich *et al.,* 2001). The *Mbd2* protein is a component of the MeCP1 protein complex and has been shown to bind to methylated DNA and repress transcription. MeCP1 is a large protein complex of 400-800 Kda which binds 12 or more methylated CpG repeats (Boyes and Bird 1992). This is thought to reflect large methylated CpG islands. Unexpectedly *Mbd2* knockout mice have no obvious phenotype apart from *Mbd2* null females producing smaller litters (Hendrich *et al.,* 2001). However fibroblast tail lines from these mice show a 25% reduction in transcriptional repression (Guy *et al.,* 2001, Hendrich *et al.,*

2001). Surprisingly there is no additive phenotype when the *Mbd2* deficient mice are crossed to *MeCP2* deficient mice (Guy *et al.*, 2001). *Mbd3* has remarkable sequence identity to *Mbd2* (75%) though it is the only MBD protein that does not bind methylated DNA (Hendrich and Bird 1998) *Mbd3* is part of the NuRD corepressor complex which has been shown to silence a wide varitey of genes in many different organisms (Ahringer *et al.*, 2000). Deficiency in *Mbd3* (unlike *Mbd2*) produces embryonic lethality by day 9. Due to the sequence similarity of the proteins it was predicted that there may be some genetic interaction between *Mbd2* and *Mbd3*. This was shown through careful analysis of breeding data as there is a deficiency of ($Mbd2^{-/-}$, $Mbd3^{+/-}$) mutants compared to ($Mbd2^{+/+}$, $Mbd3^{+/-}$) mice (Hendrich *et al.*, 2001). However the precise basis for this remains unclear.

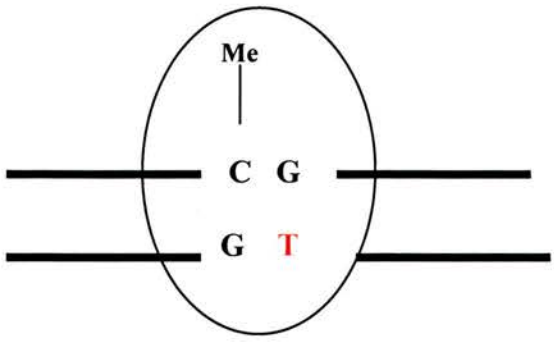
Mbd1 has also been shown to be involved in transcriptonal repression of methylated genes (Ng *et al.*, 2000). This protein can bind single methylated CpG and is not in the MeCP1 complex.

This chapter will focus on *Mbd4* deficient mice. *Mbd4* had been shown to be capable of binding single methylated CpG *in vitro* (Hendrich and Bird 1998). Unlike the other *MBD* proteins it is not involved in transcriptional repression. Two different activities have been attributed to *Mbd4*: a thymine glycolase activity and an endonuclease (Hendrich *et al.*, 1999, Bellacosa *et al.*, 1999). These have led to the proposals that *Mbd4* functions:

1. Thymine Glycolase that removes deamination
2. A mismatch repair endonuclease.

As was discussed in 1.4.6, 5-methylcytosine is prone to undergoing deamination to thymine (Hendrich *et al.*, 1999). This has led to 5 methyl-CpG being underepresented in the genome (Hendrich and Bird 2000). In the normal scenario cytosine deaminates to uracil and is removed by uracil glycolyase. However if cytosine deaminates to thymine this will not be corrected by this enzyme and will instead produce a GT mismatch.

Figure 8.1 Mbd4 binding to a deaminated 5 methyl cytosine.



MBD4

The red T shows the product of 5 methyl-cytosine deaminated to Thymine.

Although Mbd4 has been shown to bind single methylated CpGs as well as GT mismatches, it was shown to preferentially bind the structure presented in figure 8.1 (Hendrich *et al.,* 1999). It was also shown to be able to bind GU mismatches. After binding the mismatch, Mbd4 then removes the deaminated thymine using its C-terminal thymine glycolase domain. As this GT lesion would be produced post replication, it is difficult to predict whether it could be recognised by the MMR machinery. More poignantly it is doubtful whether MMR proteins would remove the incorrect T or the G as there would not be a newly synthesised strand.

MBD4 was also identified through its interaction with MLH1 in a yeast two hybrid screen. Bellacosa *et al.,* (1999) identified a weak endonuclease activity in this MLH1 interacting protein and thus called MBD4, MED1 (methyl-CpG-binding endonuclease). Bellacosa *et al.,* (1999) then proposed that due to this interaction with the MMR protein, its ability to bind methylated DNA and its endonuclease activity this made it a potential homologue of the E.coli MMR protein MutH. One outstanding question concerning mammalian MMR is how strand specificity is attained. In *E.coli* this is achieved through GATC restriction

sites in the DNA. The parental strand is methylated at these sites and thus MutH endonuclease nicks the newly synthesised unmethylated strand (Modrich 1991).

Although this is an attractive proposal, it is highly unlikely. *Mbd4* preferentially binds to the M5CpG.TpG mismatches rather than a general 5mCpG sites, thus it is involved in the repair of the mismatches rather than any general strand specific role. Given that 5mCpG is also under-represented in the genome due to this deamination and many promoters have large methylation free CpG islands, it is hard to envisage that MBD4 is a functional Mut H homologue. In addition, the weak endonuclease activity discovered by Bellacosa *et al.,* (1999), was not found in the study of Hendrich *et al.,* (1999). This indicates that this may not be of prime importance to the protein. Indeed, Drummond and Bellacosa (2001) recently showed that in HeLa cells MMR works independently of CpG methylation. However its ability to bind to *Mlh1* and these GT mismatches, does implicate that it may play a role in MMR. Another potential scenario is that *Mbd4* interacts with the MMR proteins to stop them binding at these mismatches to prevent incorrect removal of the guanine.

MBD4 has also been implicated as a tumour suppressor gene in the intestine. *MBD4* has been shown to be mutated in human colorectal cancers characterised with microsatellite instabilty (Riccio *et al.,* 1999, Bader *et al.,* 1999, 2000). Bader *et al.,* (2000) suggested that up to 89% of sporadic colorectal cancers with MSI had a *Mbd4* mutation. However the significance of this is uncertain as nearly all of the mutations are in the poly A10 tract in the human M*bd4* gene (Riccio *et al.,* 1999, Bader *et al.,* 1999, 2000). Therefore this could just be a marker of mismatch repair deficiency and MSI.

To investigate the significance of *Mbd4 in vivo*, *Mbd4* deficient mice were generated by Jacky Guy of the Adrian Bird laboratory. These mice were viable and had no obvious tumour predisposition. To address the possibility that *Mbd4* may function as a MMR protein, the $Mbd4^{-/-}$ mice were therefore characterised *in vivo* for mutation frequency, apoptosis and accelerated $Apc^{Min/+}$ neoplasia.

The results obtained show that the activity of *Mbd4* cannot be explained through its interaction with MMR proteins and instead establish a fundamental role for *Mbd4* in the cellular response to DNA damage and in suppression of intestinal neoplasia.

8.1 Results

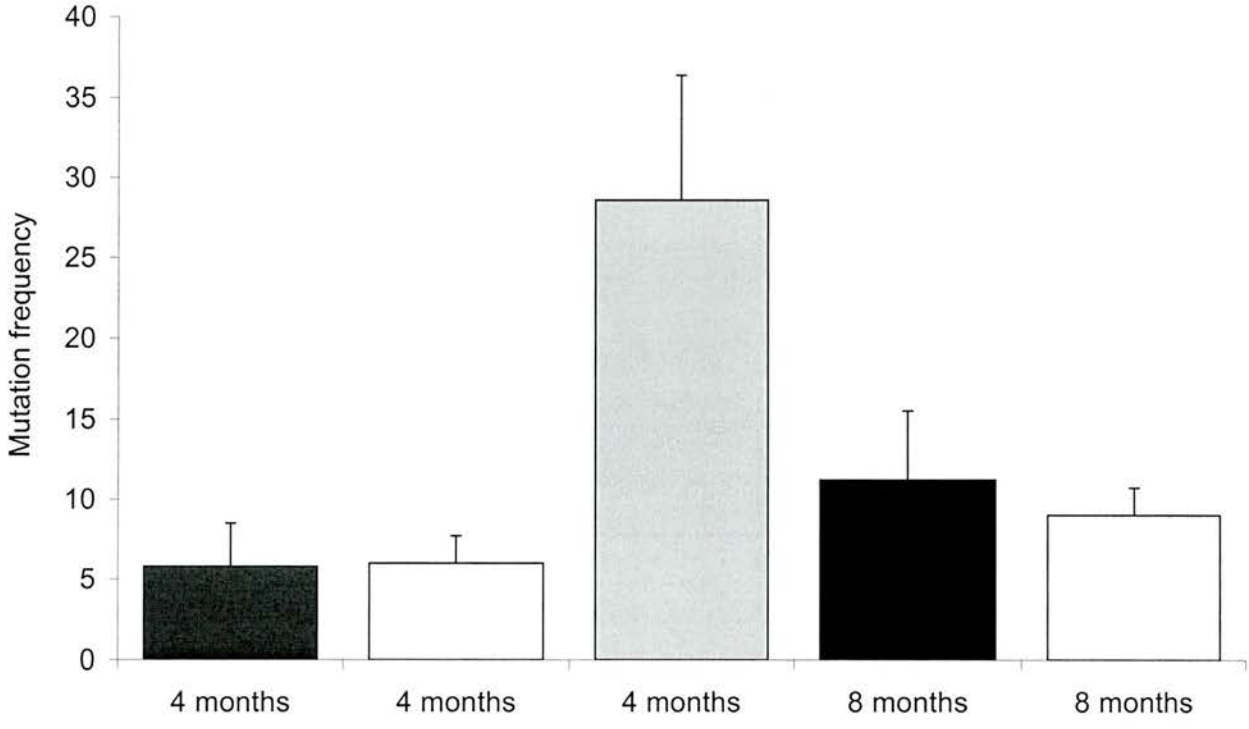
Previous studies have demonstrated an interaction between *Mbd4* and the mismatch repair gene *Mlh1*, an interaction which suggests a potential role for *Mbd4* in recognising mismatch type damage. To determine if this interaction was specific to *Mlh1* or could be generalised to other DNA damage associated proteins Catherine Millar performed a series of immunoprecipitations using Hela cell extracts. These confirmed an interaction with *Mlh1*, but also show an interaction with *Msh2* though not *Pms2* and *Msh6*. This argues against *Mbd4* being part of the functional MMR complex. Notably these interactions occur through the C-terminal domain of *Mbd4*, rather than through the methyl binding domain (MBD)

To determine the functional significance of *Mbd4* deficiency Jacky Guy generated mice bearing a null *Mbd4* allele. Mice heterozygous for this allele were intercrossed and homozygotes generated. Mice homozygous for the mutation were detected at the expected Mendelian ratio, demonstrating that deficiency for *Mbd4* was compatible with viability. Functional inactivation of *Mbd4* was confirmed in the homozygotes using both Quantitativive PCR and western analysis. The null mice show no overt phenotype and are not prone to the development of spontaneous malignancy, at least up the age of 18 months.

8.1.1 Spontaneous mutation frequency in Mbd4 deficient mice

As was mentioned in 8.1, three different lines of evidence suggest that *Mbd4* may modulate the mutation frequency *in vivo*. First, that *Mbd4* functions as a thymine glycosylase at CpG sites and therefore deficiency of *Mbd4* may increase the rate of spontaneous deamination. Second, that *Mbd4* directly interacts with the MMR proteins *Mlh1* and, as we show here, *Msh2*. Third, it has been suggested by Bellacosa *et al.,* (1999) that *Mbd4* is an endonuclease and as such it has been proposed as a potential candidate for the eukayotic homologue of the MutH endonuclease. Taken together, these findings predict that loss of *Mbd4* would result in elevated mutation, and that *Mbd4* deficiency may parallel the mutator phenotype of MMR deficiency. Therefore mutation frequency was scored in the intestinal epithelium using the *Dlb-1b* assay in both normal and *Mbd4*$^{-/-}$ mice.

Figure 8.2: No increased mutation in *Mbd4* deficient mice. Mutation frequency at the *Dlb1-b* locus per 10000 villi. Black bars, wild type mice; open bars, *Mbd4*$^{-/-}$ mice; Grey bars, *Msh2*$^{-/-}$ mice. By 8 months nearly all *Msh2*$^{-/-}$ mice are dead thus mutation was not scored in these mice

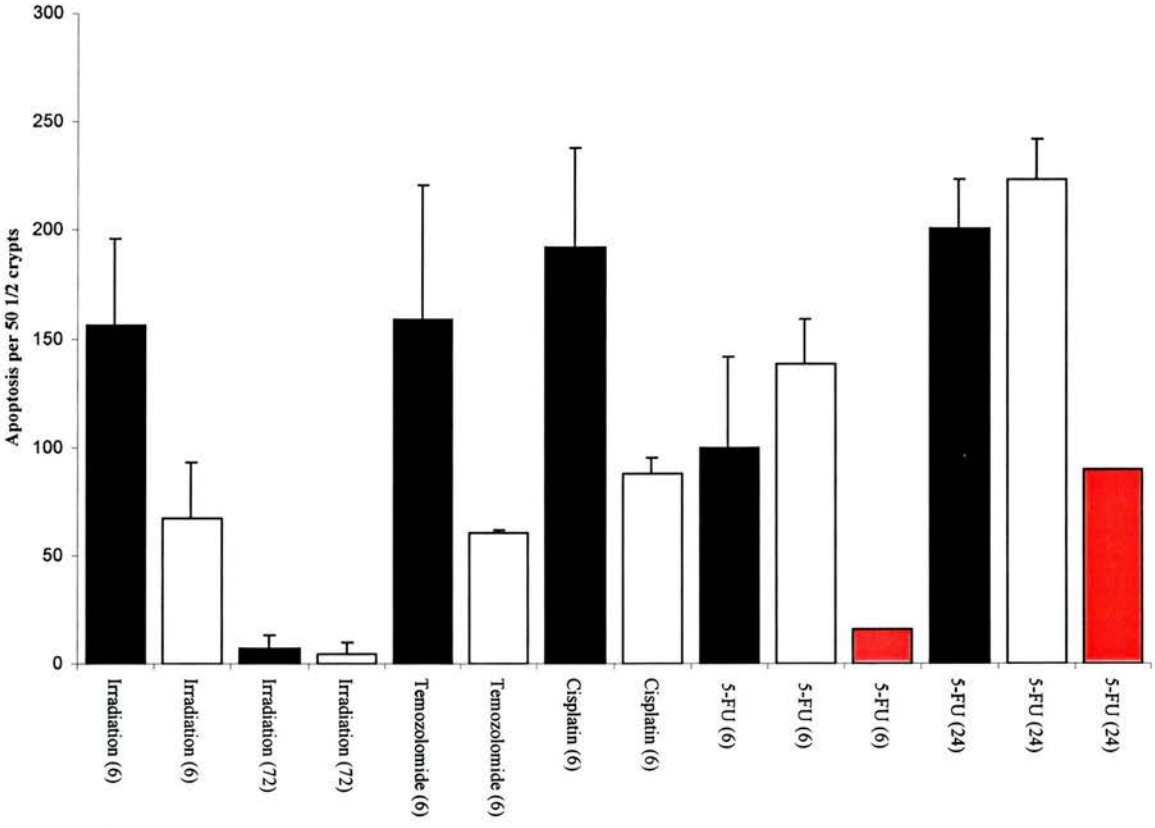


Using this assay, which reports mutation *in vivo* within the stem cell compartment of the crypts of Lieberkuhn, no increase in spontaneous mutation frequency in *Mbd4* null mice was found. This result argues that *Mbd4* is not essential for MMR *in vivo*.

8.1.2. Reduced levels of apoptosis in *Mbd4*$^{-/-}$ mice

The mismatch repair proteins *Mlh1* and *Msh2* have also both been shown to influence the apoptotic response to DNA damage. Therefore influence of *Mbd4* status upon the apoptotic response of intestinal enterocytes was analysed at both 6 hours and 72 hours following exposure to DNA damage.

Figure 8.3: Apoptosis per 50 ½ crypts in wild type and *Mbd4* deficient following a range of DNA damaging agents. Black bars, wild type mice; open bars, *Mbd4*$^{-/-}$ mice and pink bars, *p53*$^{-/-}$ mice. Error bars represent SD.

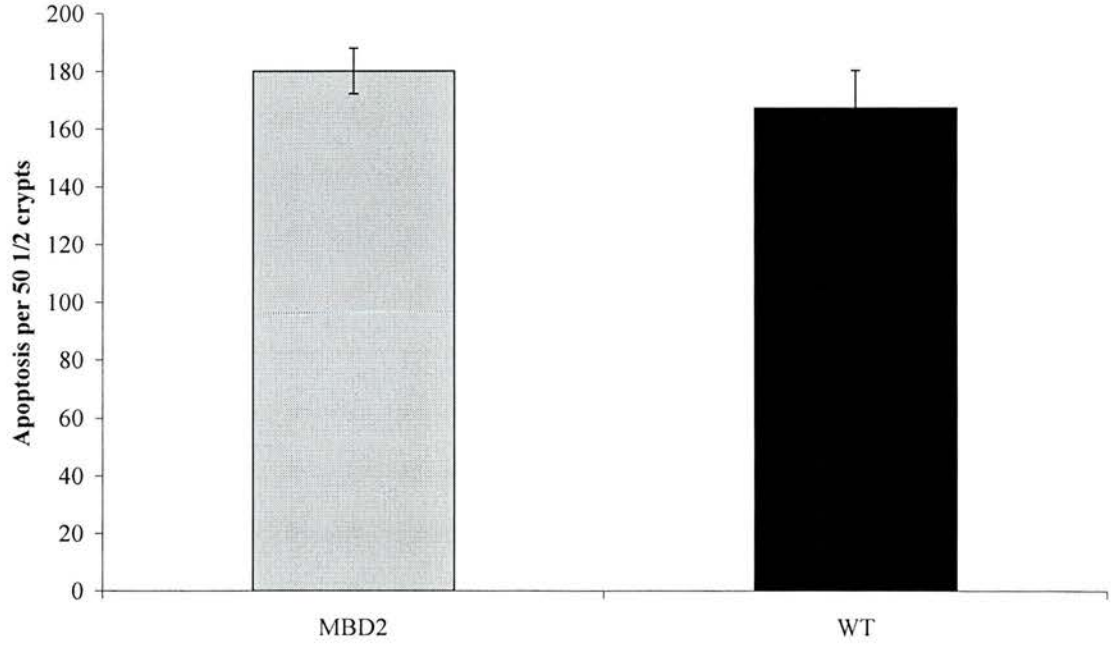


Mice null for *Mbd4* show a normal apoptotic response following exposure to the pyrimidine antimetabolite 5-fluorouracil (5FU) at either 6 or 24 hours (p=0.32 and p=0.10, Mann Whitney), but a markedly reduced apoptotic response following exposure to ionising radiation (p=0.001, Mann Whitney), cisplatin (p=0.04, Mann

Whitney) and the alkylating agent Temzolomide (p=0.04, Mann Whitney) (figure 8.2). These data imply that *Mbd4* is specifically required for signalling apoptosis from DNA damage, as 5FU has been shown to mediate apoptosis through changes in RNA metabolism rather than by DNA damage (Pritchard et al 1997,1998). The failure of 5FU to elicit *Mbd4* dependent death is also notable in the light of reports that *Mbd4* can bind and remove 5-FU damage (Petronzelli *et al.,* 2000). These results demonstrate a wide ranging role for *Mbd4* in mediating cell death.

To test if the apoptotic phenotype was generic to all proteins containing a methyl binding domain, the apoptotic phenotype of mice deficient for *Mbd2* following ionising radiation was analysed and found them not to differ from controls was also analysed.

Figure 8.4: Normal Apoptotic response following gamma irradiation in *Mbd2*$^{-/-}$ mice. Apoptotic response per 50 ½ crypts following 5 GY of irradiation. Black bars, wild type mice, Grey bars, *Mbd2*$^{-/-}$ mice.

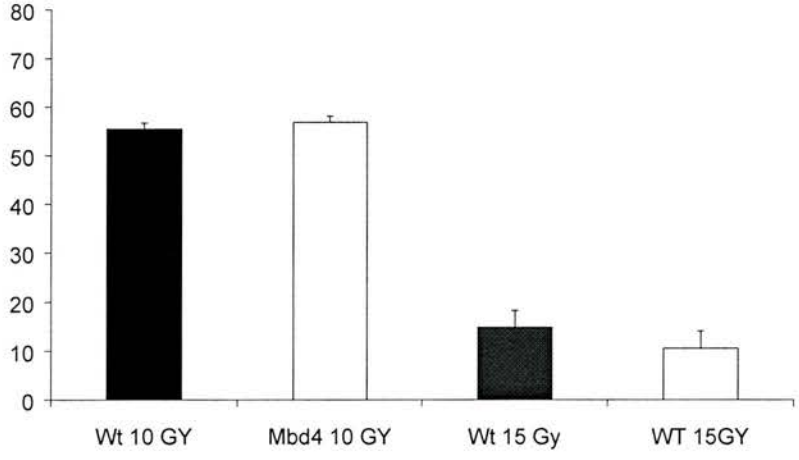


8.1.3 Clonogenic survival and induced mutation frequency in *Mbd4*$^{-/-}$ mice

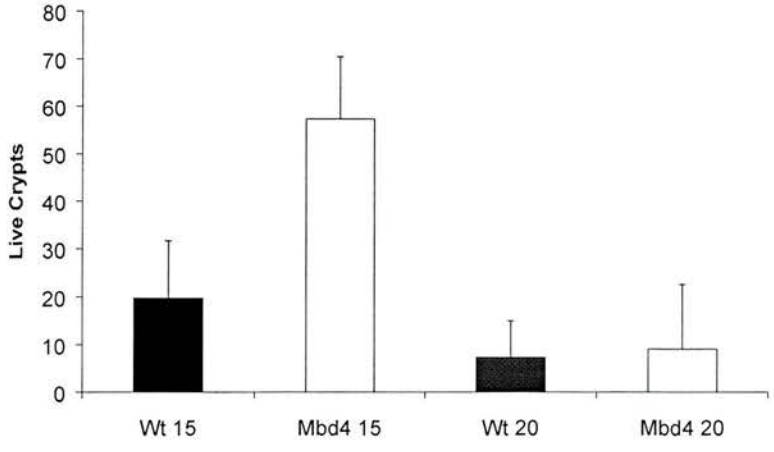
The observed reduction in the apoptotic response of *Mbd4* mutant mice implies that cells fail to be appropriately deleted in the absence of *Mbd4*. This in turn argues that clonogenic survival and induced mutation frequency at the *Dlb1-1b* locus would be increased in the *Mbd4* null mice following these agents.

Figure 8.5: Clonogenic survival in wild type and *Mbd4*$^{-/-}$ mice following gamma irradiation and cisplatin. Average number of surviving (live) crypts per circumference of small intestine. Black bars, wild type mice; open bars, *Mbd4*$^{-/-}$ mice.

A) 10 and 15Gy γ Irradiation

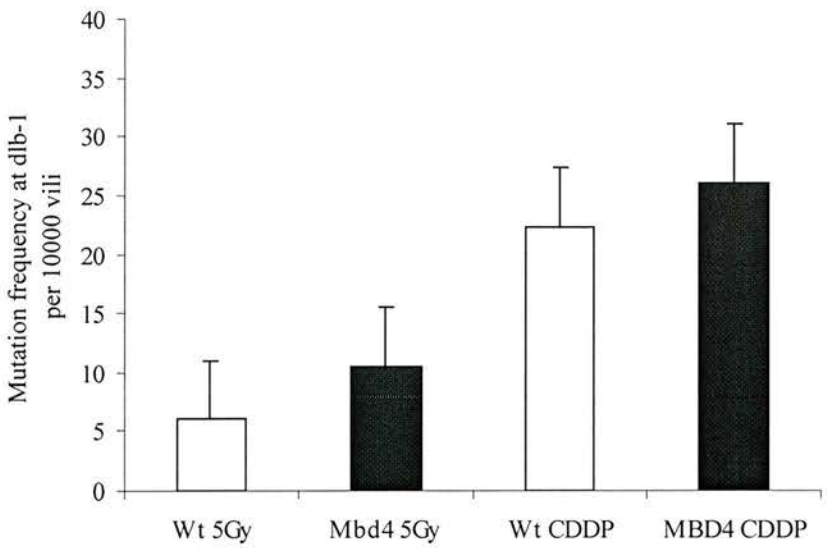


B 15mg/kg and 20mg/kg Cisplatin



Mbd4 deficiency does not lead to increased clonogenic survival following exposure to exposure to gamma radiation (p=0.4, Mann Whitney), but does but does cause increased survival following exposure to cisplatin (p=0.01, Mann Whitney). Due to this increase in survival following cisplatin, mutation frequency was then scored (figure 8.6).

Figure 8.6: Induced mutation frequency following 5 Gy γ-irradiation and 5mg/kg cisplatin

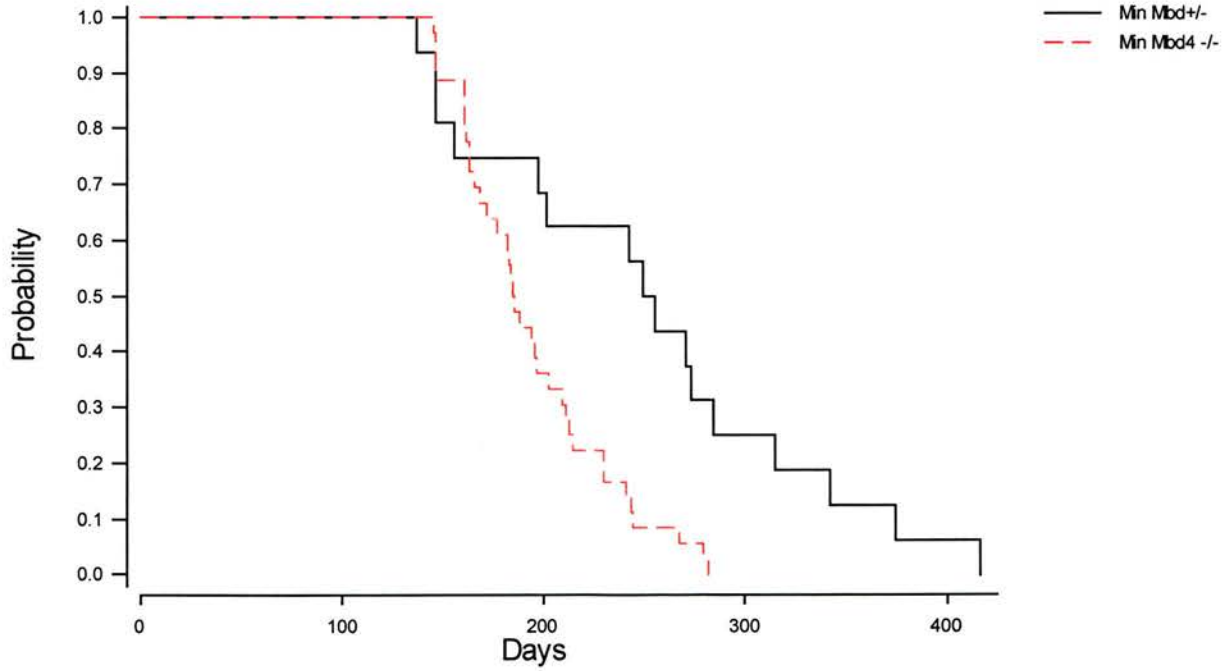


Despite the damage specific nature of the clonogenic response, no *Mbd4*-dependent increase in mutation frequency was found following exposure to either agent (p=0.4 (Ionising radiation) and p=0.65, Mann Whitney (Cisplatin)). These results again parallel data obtained from *p53* deficient animals, which show that although *p53* functions as a robust tumour suppressor gene and is essential for the normal induction of apoptosis, deficiency of *p53* does not necessarily lead to a marked increase in mutation frequency or clonogenic survival.

8.1.4. *Mbd4* deficiency accelerates intestinal neoplasia on an $Apc^{Min/+}$ background

Mbd4 has previously been implicated as a putative tumour suppressor in the intestine (Bader *et al.,* 1999, 2000, Riccio *et al.,* 1999). To directly test if *Mbd4* deficiency can alter the predisposition to malignancy mice deficient for *Mbd4* were crossed onto an Apc^{Min} heterozygous background. This background predisposes to the development of spontaneous neoplasia within the intestine. Littermates were divided into two cohorts, either ($Mbd4^{-/-}$, $Apc^{Min/+}$) (n=36) or ($Mbd^{+/-}$, $Apc^{Min/+}$) (n=17). These cohorts were closely monitored and animals killed when they became overtly ill.

Figure 8.7: Kaplan Meier survival plot of ($Mbd4^{-/-}$, $Apc^{Min/+}$) and ($Mbd4^{+/-}$, $Apc^{Min/+}$). Black solid line, ($Mbd4^{+/-}$, $Apc^{Min/+}$) mice, red dashed line ($Mbd4^{-/-}$, $Apc^{Min/+}$) mice.



Comparison of the survival curves showed that deficiency of *Mbd4* markedly reduced survival (p=0.001 Log Rank). Tumour incidence within the large and small intestine was scored at necropsy, with (*Mbd4*$^{-/-}$, Apc$^{Min/+}$) (having higher number of tumours (p=0.02) (figure 8.9). Histological analysis showed no difference in the morphological appearance of lesions between the cohorts.

Figure 8.8: Large intestine from (*Mbd4*$^{-/-}$, *Apc*$^{Min/+}$) mouse showing colorectal adenomas.

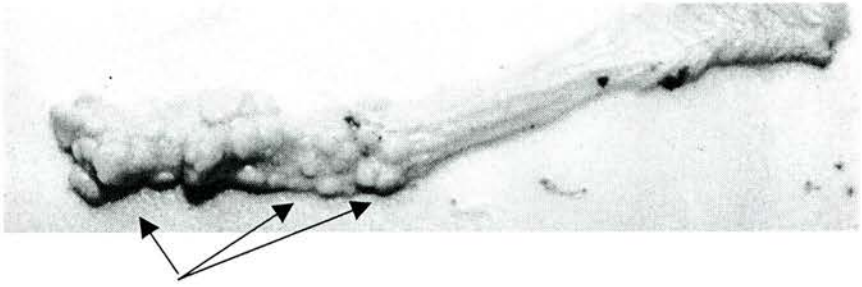


Figure 8.9: Median Tumour burden.

	Small Intestine	Large Intestine	Total
(*Mbd4*$^{+/-}$, Apc$^{Min/+}$)	3	11	14
(*Mbd4*$^{-/-}$, Apc$^{Min/+}$)	11	17	28

These findings indicate that tumour development was accelerated in the absence of *Mbd4*, but that *Mbd4* deficiency did not alter the phenotype of lesions arising.

8.2 Discussion

This comprehensive analysis of the *in vivo* consequences of loss of function of *Mbd4* has shown that it interacts with a series of MMR proteins, that it modifies the apoptotic response and that deficiency of *Mbd4* accelerates tumorigenesis in a murine model of human FAP.

The significance of the interaction with MMR genes however is still unclear. Bellacosa *et al.,* (1999) showed that over-expressing a mutant MBD4 protein caused increased MSI. However this mutant still contained its MLH1-binding domain, thus it could cause this mutator phenotype by binding to MLH1 and preventing it function in MMR. In fact over-expression of MLH1 has been shown to cause a mutator phenotype in yeast (Schberberkova and Kunkel 1999) probably by inappropriate formation of MLH1 homodimers. Underlining the fact that one should be wary of over-interpreting of over-expression studies.

The failure to see a mutator phenotype in the *Mbd4* deficient mice argues strongly against it being involved in MMR. We have shown that loss of *Mlh1*, *Msh2* and *Pms2* all produce an increased spontaneous mutation frequency at the *Dlb-1b* locus. With respect to the role of *Mbd4* in repair of deamination at 5-methylcytosine sites, the level of mutation resulting from these events may also be below the threshold of detection by our assays. Alternatively, mouse cells possess at least one other T-G glycosylase activity, TdG (Neddermann, 1996), which may in theory compensate for loss of *Mbd4*.

The interaction with the MMR proteins cannot explain the apoptotic phenotype seen in the *Mbd4* deficient mice as the deficiency in apoptosis in the *Mbd4*$^{-/-}$ mice are more marked and wide ranging that the MMR mice. As was shown in chapter 5 there was only a small reduction in the apoptotic response to cisplatin in *Msh2* $^{-/-}$ mice. Currently there is no data showing that *Msh2* plays a role in mediating the apoptotic response to γ-irradiation.

One interesting hypothesis is that the interaction between MMR and *Mbd4* is a negative one. The basis for this is that, if MMR bound the M5CpG.TpG mismatches there would be no indicator for strand specificity, as this is a deamination event rather than a replication error. Thus it is likely that 50% of the repair by MMR would result in a mutation (Bellacosa *et al.,* 1999).

As the *Mbd4* dependent apoptosis cannot be explained through the interaction with the MMR proteins, this leaves the mechanism of *Mbd4* mediated apoptosis rather unclear. Although, Catherine Millar was unable to pull down any other proteins with *MBD4* in the HeLa cells, this was experiment was performed using untreated cells so it will be interesting to see what *MBD4* interacts with post DNA damage.

As *Mbd4* responds to such a wide range of damaging agents it is clearly of fundmental importance in signalling death. The 5-FU experiment indicates at *Mbd4* is important for signalling apoptosis from DNA damage rather than all types of apoptotic stimuli.

Mbd4 has been shown to bind the structure in fig 8.1, mismatches and methlylated CpG, thus apart from possibly binding O6meG lesion produced by temozolomide, it is unlikely that it would be able to bind damage from all these types of lesion. Therefore it appears that it is involved in signalling apoptosis rather than damage recognition.

Fishel (pers comm) have shown using GST tagged MBD4 interacts with a range of DNA repair proteins: BRCA1, BRCA2, MRE11, RAD51 and RAD52. These proteins have all been shown to be important for signalling the presence of double strand breaks. In fact these are part of the BASC (BRCA1 associated super complex) isolated from HeLa cells which was shown to be upregulated after a range of DNA damaging agents (Wang *et al.*, 2000). As cisplatin, gamma irradiation and temozolomide all induce strand breaks this produces a model where *Mbd4* is part of a much larger complex which signals apoptosis. Loss of

Mbd4 could therefore make this complex less efficient at signalling death. Another model would be that *Mbd4* interacts with this complex to signal death.

Catherine Millar was unable to show these interactions in Hela cells, however as was mentioned this was using untreated HeLa cells. Therefore further work is required to characterise the mechanism of *Mbd4* dependent apoptosis. At present an *Mbd4* antibody is being generated for mouse, therefore the kinetics of the *Mbd4* response following DNA damage can be investigated. For example, it will be possible to determine whether *Mbd4* is induced following DNA damage. This would also allow an examination of which proteins *Mbd4* interacts with in the murine intestine through co-immunoprecipations with *Mbd4* following DNA damage.

As was shown in chapter 5 and many times in the literature, this reduction in the apoptotic response does not necessarily lead to increased survival and mutation (Brown and Woulters 1999). In fact, the increased clonogenic survival following cisplatin treatment is one of the rare instances where a reduced apoptotic response correlates with increased survival. In this thesis, both *Mbd4* and p53 deficiency causes increased survival following cisplatin damage. *Mbd4* deficiency only produces increased survival at 15mg/kg of cisplatin, whilst *p53* deficient animals showed increased survival at both 15mg/kg and 20mg/kg. Care should be taken against over-interpreting the direct comparison of these results as the *Mbd4*$^{-/-}$ animals were maintained on a C57Bl/6 background and the *p53*$^{-/-}$ mice were maintained on an outbred background. It has previously been reported that levels of crypt killing is dependent on background and this is confirmed here as there are very different levels of death in the C57Bl/6 wild type mice used in the *Mbd4*$^{-/-}$ experiment and the outbred wild types used in the *p53* experiment (Potten, 1990).

As with *p53* deficiency, there was no increase in survival in *Mbd4*$^{-/-}$ mice following gamma irradiation. One possible explanation is that the very high levels of damage that is needed to kill crypts in the microcolony assay could saturate out any gene dependent difference. However when lower levels of

damage are used for the mutation induction experiment, there was no increase in mutation at the *Dlb-1b* locus in the *Mbd4* nulls above wild type levels after either gamma irradiation or surprisingly cisplatin. Therefore this once again this dissociates loss of apoptosis from mutation induction.

Clarke *et al.,* (1997) have previously shown that mutation frequency in $p53^{-/-}$ mice was not significantly higher than wild types levels after 5GY of irradiation although there was a significant increase after 7.5GY. However there was no difference in the levels of apoptosis induced by these two doses of gamma irradiation. It would be interesting to see whether there is an increase in mutation frequency following 7.5Gy irradiation in the $Mbd4^{-/-}$ mice.

As *Mbd4* has been shown to signal apoptosis it is currently being examined whether it also signals cell cycle arrest (via BRDU incorporation) following DNA damage in the small intestine. A more detailed study of cell cycle checkpoint analysis will then be performed in primary fibroblasts and and compared to colorectal cell lines. Bader *et al.,* (1999,2000) have shown that there are a number of colorectal cell lines available that have lost *MBD4* thus these could be used.

MBD4 has been described by Riccio et al (1999) as a tumour suppressor, due to its loss in many mismatch repair deficient tumours. However this could easily be a reflection of MSI in the MMR tumours as most of the mutations in the *Mbd4* gene are at the A(10) tract in its coding sequence (Bader et al 1999, 2000). Here we show *Mbd4* deficiency per se does not lead to a spontaneous tumour predisposition. However it does accelerate intestinal neoplasia in the $Apc^{Min/+}$ mouse, a murine model of FAP. Therefore this confirms the role of *Mbd4* as a tumour suppressor. We have shown that there is no obvious increase in mutation. Therefore, whilst the possibility remains that *Mbd4* is involved in both damage signalling and repair of the genome, the data presented here implicate the apoptotic signalling role of *Mbd4* in tumour suppression.

As yet no mutations have been found in *MBD4* in FAP nor sporadic colorectal cancer without MSI (Bader *et al.*, 2000). There are two potential reasons for this, first that *MBD4* has not been screened thoroughly for mutation and second, that unlike the A10 tract there is not a putative mutation hotspot. In tumours without MSI, SSCP (Single Stranded Conformation polymorphism) which could potentially miss some of the mutations in *MBD4* (Bader *et al.*, 1999). However the most plausible explanation is that *Mbd4* is not loss in these tumours.

Therefore despite loss accelerating the murine model of FAP, there is no evidence yet that *MBD4* is lost in FAP. This could reflect species specific difference between human colorectal neoplasia and murine intestinal neoplasia. However the fact *MBD4* is lost in the majority of microsatellite unstable tumours does imply it plays a role in intestinal malignancy in humans. It is worth remembering that *Msh2* and *Mlh1* both rapidly accelerate murine models of FAP though they are not lost in FAP.

At present, *Mbd4* deficient mice are being crossed to MMR deficient mice to see whether intestinal malignancy is accelerated in this background. This should provide definitive evidence of whether loss of *Mbd4* is selected for in a MMR deficient background or is simply a reflection of MSI.

Taken together these results argue that *Mbd4* is a modifier of intestinal tumourigenesis which when lost accelerates tumourigenesis. The chance of this loss is greatly increased in colorectal cancers with defects in its MMR due to the presence of an unstable A10 tract and has implications for chemotherapy. Given the similarities between the consequences of loss of *Mbd4* and *p53* for the apoptotic response following DNA damage, it is interesting to note that MMR deficient colorectal cancers very rarely have *P53* mutations. It was argued in chapter 6 that this could be due to under reporting of *P53* mutations, another possible interpretation is that loss of *MBD4* in these tumours circumvents the requirement of a *P53* mutation.

Chapter 9: Characterisation of β-catenin expression and apoptosis in murine models of intestinal neoplasia.

9.0 Introduction.

In the previous chapters, this thesis has examined the apoptotic response in normal intestinal epithelium. This chapter investigates whether apoptosis in adenomas exhibits the same gene dependency as the apoptosis that occurs in normal mucosa. This was done by crossing *Msh2*$^{-/-}$ deficient mice to *Apc*$^{Min/+}$ mice, and analysing the apoptotic response in these lesions.

Germline mutations in the adenomatous polyposis coli gene (APC) gene at 5q21 characterise an inherited disorder known as familial adenomatous polyposis coli (FAP) (Nishino *et al.,* 1991).

The precise nature of how loss of function of *Apc* predisposes to malignancy remains unclear, however disruption of the normal function of β-catenin has been implicated in this process (Rubinfeld *et al.,*1993, Su *et al.,* 1993).

As mentioned in 1.4.1, levels of β-catenin are modulated by APC through the mammalian Wnt signaling pathway, where APC interacts with axin, GSK3β and β-catenin. The central portion of APC contains sites at which it can be phosphorylated by glycogen synthase kinase 3β (GSK3β) and also through which it complexes with β-catenin. Phosphorylation by GSK3β increases the stability of the APC/β-catenin complex and is thereby thought to increase the rate of β-catenin degradation (Rubinfeld *et al.,* 1996) (see figure 1.2 for Wnt signalling).

Immunohistochemical analysis of both human and murine intestinal tumours has shown that both adenomas and well differentiated carcinomas are characterised by high levels of β-catenin (Inomata *et al.,*, 1996, Takayama *et al.,* 1996). However, both β-catenin and E-cadherin are reported to be expressed at significantly lower levels in more aggressive malignancies, strongly suggesting that over-expression of β-catenin is only crucial in early tumour development (Takayama *et al.,* 1996). In order to further characterise the association between tumourigenesis and

dysregulation of β-catenin, levels of β-catenin expression in normal and neoplastic tissue derived from mice mutant for the tumour suppressor genes $Apc^{Min/+}$ and *Msh2* were analysed in this chapter.

Dysregulated Wnt signalling is thought to drive transcription of a plethora of genes, many of which are oncogenes e.g *AP1*, *c-myc*, *cyclin D1* and have been shown to drive both apoptosis and proliferation depending on cellular context (Evan *et al.,* 1992). Previous studies have shown that levels of apoptosis in normal human colorectal epithelium are much higher than in adenomas and carcinomas (Bedi *et al.,* 1995). This indicates that loss of apoptosis may be important to tumour progression. This loss of apoptosis may be a relatively early event, as when Fazeli *et al.,* (1997) compared levels of apoptosis from adenomas to adenocarcinomas (see figure 1.1), they found no differences in the levels of apoptosis. This also suggests that the early reduction in the apoptotic is independent of *P53* status as loss of *P53* is most frequently reported at the adenoma-carcinoma transition (Vogelstein and Kinzler, 1996).

Both the $Apc^{Min/+}$ and the ($Apc^{Min/+}$, $Msh2^{-/-}$) models of intestinal neoplasia possess the great advantage of developing multiple lesion types within the same mouse. Thus, a spectrum of lesions can be seen from single dysplatic lesions to (very rarely) large adenocarcinomas within the same animal (Clarke *et al.,* 1995). Therefore the stage where apoptosis is lost can be accurately characterised

One future aim of chemotherapy is tailoring of the genetic profile of the tumour to therapy: i.e. if a certain gene is lost, a certain therapy will or will not work. In chapter 4, reduced levels of apoptosis were shown in $Msh2^{-/-}$ mice following temozolomide treatment (also Toft *et al.,* 1999). Therefore whether this gene dependency is retained in tumours will be assessed by comparing tumours from ($Apc^{Min/+}$, $Msh2^{+/+}$) to ($Apc^{Min/+}$, $Msh2^{-/-}$) mice.

9.1 Results

9.1.1 Expression of β-catenin in intestinal tumours.

The pattern of β-catenin expression in intestinal lesions developing within $Apc^{Min/+}$ mice was investigated. Mice were harvested when they had intestinal neoplasia and the β-catenin staining was performed by R. Kongkanuntn. In morphologically normal epithelium, β-catenin was localised at the cell periphery or in the nucleus. Nuclear localisation was also observed in some cells: these were always located at the crypt base (figure 9.1) The expression of β-catenin was significantly more intense in dysplastic crypts and small adenomas. To control for staining variability between sections, changes in the intensity of expression were always scored relative to normal epithelium within the same section. The lesions were subclassified as in Clarke *et al.,* (1995): (I) single dysplastic crypts, showing nuclear pleomorphism and stratification; (II) complex lesions, comprising several architecturally distorted crypts in the lamina propria with virtually normal overlying surface epithelium; (III) small adenomas, identified by the overall disturbance of architecture including the surface and distinguished from the previous category on the basis of increased size and surface involvement; (IV) large adenomas, and (V) invasive adenocarcinoma.).

Figure 9. 1. β-catenin expression patterns within intestinal lesions. Percentage of each lesion type showing either 100% upregulation of β-catenin (+ve); a mosaic or heterogeneous pattern of upregulation as defined in the text (M); or no upregulation (-ve). N indicates the number of lesions scored.

	Lesion Type														
	I			II			III			IV			V		
	+ve	M	-ve	+ve	M	-ve	+ve	M	-ve	+ve	M	-ve	+ve	M	-ve
Apc +/-;	56	44	0	58	42	0	58	42	0	55	45	0	60	40	0
	N=41			N=96			N=36			N=20			N=10		
Apc +/-: *Msh2* -/-	70	27	3	68	27	5	50	47	3	60	40	0	50	50	0
	N=187			N=120			N=30			N=5			N=4		
Msh2 -/-	20	20	60	17	17	66	56	33	11	50	50	0	0	0	0
	N=5			N=6			N=9			N=8			N=0		

The pattern of β-catenin staining is summarised in figure 9.1. A substantial proportion of all lesion types showed heterogeneous expression of β-catenin expression, even where only single crypts were involved (type I lesions, fig 9.2b,9.2e). The term 'heterogeneous' is used here to describe lesions in which only a proportion of cells were characterised by increased expression. Although heterogeneous β-catenin was observed in all lesions types, the proportion of cells overexpressing β-catenin were at their highest in type I, II and III lesions, even where the level of histological atypia was slight (fig 9.2c-d). Type IV-V lesions showed a lower proportion of cells staining positive for β-catenin (fig 9.2f), with large areas of reduced staining observed in some lesions (fig 9.2g).

In all categories of lesion the pattern of β-catenin staining was predominantly nuclear. Strong nuclear localisation was observed within some lesions as has been previously reported (Sheng *et al.,* 1998). This pattern of localisation of β-catenin should be taken with some caution, as it has been observed that there can be a fixation dependent alteration in the apparent intracellular location of β-catenin (Kongkanuntn *et al.,* 1999). Although a similar approach did not show fixation

dependent differences in intestinal material, it remains possible that an element of the cytoplasmic staining observed here arises as a consequences of either fixation or processing protocols.

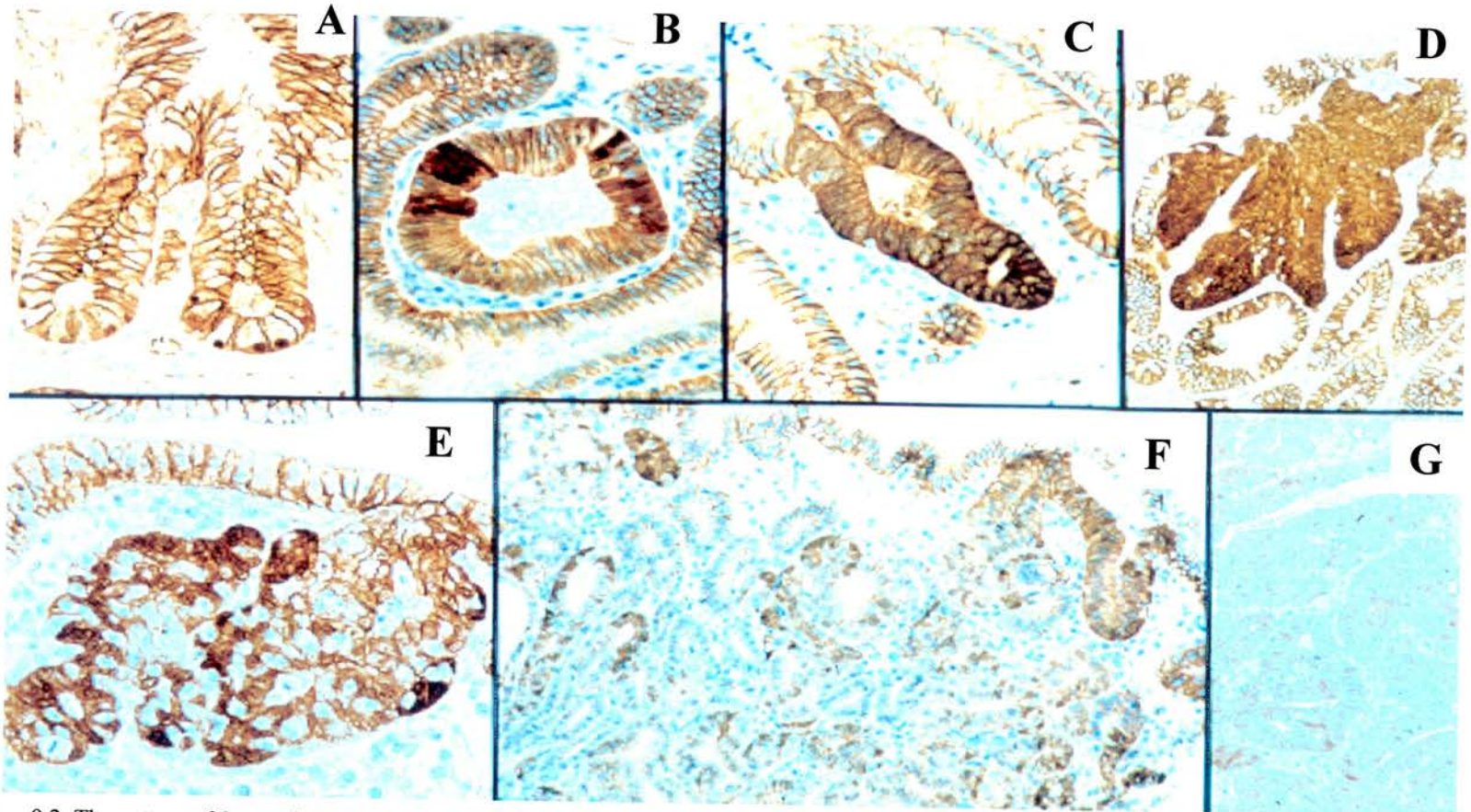
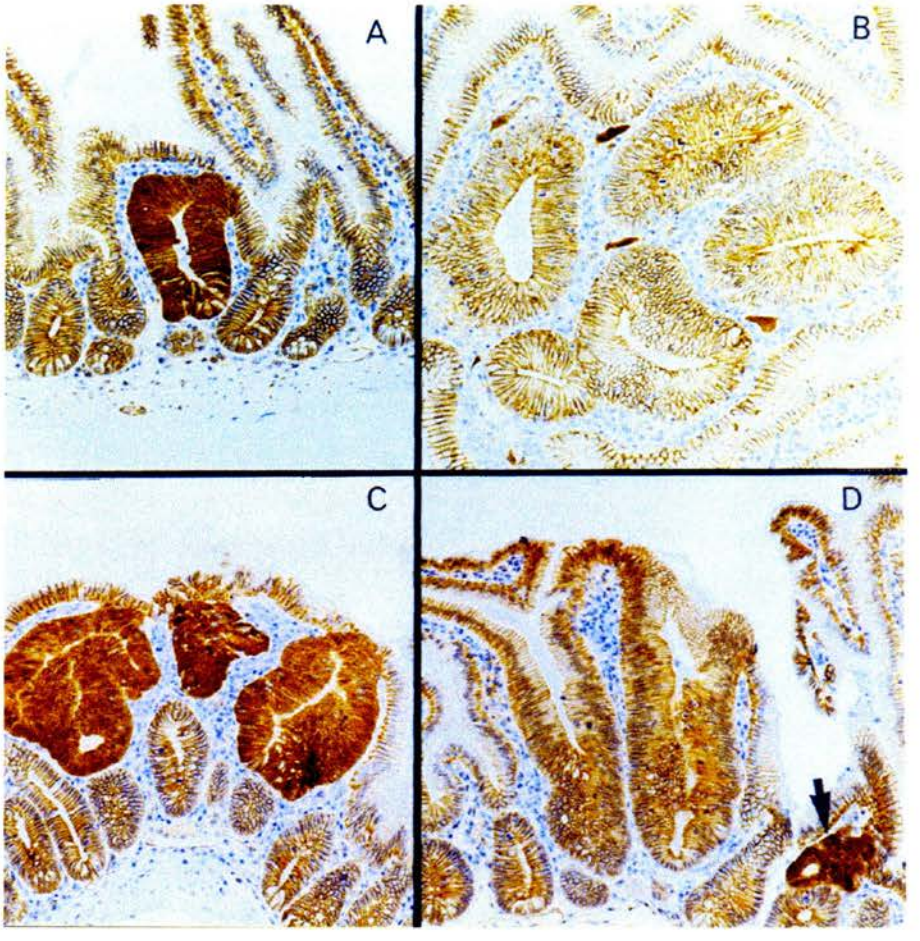


Figure 9.2. The pattern of β-catenin staining in the intestine of Apc^{Min} +/- and (Apc^{Min} +/-, p53 -/-) animals. (A-G) Photographs demonstrating the various features observed in animals with these genotypes. All the features illustrated here were observed irrespective of p53 status. All scale bars represent 10uM A) β-catenin staining in morphologically normal crypts of the small intestine. β-catenin was detected throughout the cytoplasm of epithelial cells but was strongly localised to the lateral borders. Strong nuclear localisation was observed in cells at the crypt base (arrows) B) Heterogeneous expression in a type I lesion. The majority of cells show the normal pattern of staining, with localisation to the lateral borders. A subset of cells show increased cytoplasmic and nuclear staining. C) Uniformly increased β-catenin staining within a type 1 lesion. D) Increased β-catenin staining in a type II lesion. E) Heterogeneous expression in a type II lesion. Cells showing increased β-catenin showed localised to the cytoplasm and in some instances localisation to the nucleus F) Heterogeneous expression of β-catenin within a type III lesion. G) Reduced expression within a type IV lesion. Where expression of β-catenin was retained this was often localised to the nucleus.

9.1.2 Expression of β catenin in intestinal tumours from *Msh2*$^{-/-}$ and (Apc$^{Min+/}$, Msh2$^{-/-}$) mice

The pattern of β-catenin expression in *Msh2*$^{-/-}$ and (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) animals is summarised in 9.1). Previous studies have shown that the *Msh2* mutation predisposes to intestinal tumorigenesis and also accelerates neoplasia in *Apc*$^{Min/+}$ mice (Reitmair *et al.,* 1996A., De Wind *et al.,* 1995, De Wind *et al.,* 1998). In *Msh2*$^{-/-}$ animals there were type I, II and type III lesions which showed normal β-catenin expression (fig 9.3b), a phenomenon that was not observed in *Apc*$^{Min/+}$mice (fig 9.3a). However, the majority of type I and II lesions and all type III adenomas were characterised by increased levels of β-catenin expression. In (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) mice there was a significant increase in the frequency of adenomas, as has been previously reported (Reitmair *et al.,* 1996A). The majority of these lesions stained strongly for β-catenin (fig 9.3c), however there were again a small number of type I and II lesions (<5%) with the wild type pattern of β-catenin expression (fig 9.3d). Only 1/35 type III lesions showed normal β-catenin expression.

Figure 9.3 The pattern of β-catenin expression in *Msh2*$^{-/-}$ mice (A-B) and (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) mice (C-D). All scale bars represent 10uM A) Increased β–catenin staining in a type I lesion. B) Normal β-catenin expression in a type II lesion., with β-catenin strongly localised to the lateral borders. C) Increased β-catenin expression in a type II lesion.. D) Normal β-catenin expression in a type II lesion, with retained localisation to the lateral borders. A type I lesion showing β-catenin dysregulation is indicated for comparison (arrow).



9.1.3. Expression of Cyclin D1 in the intestinal neoplasia

Due to the increased levels of β-catenin in the intestinal lesions of the $Apc^{Min/+}$ mice, one prediction was that targets of β-catenin TCF/LEF transcription should also be over-expressed. One potential target of Wnt signalling is cyclin D1 which was identified as by overexpressing mutant β-catenin (non-regulatable) in colorectal cell lines and showing that levels of cyclin D1 increased (Tetsu and McCormick 1999). Notably, these colorectal cells lines would have already had mutations in the *Apc* gene so it is quite surprising that they were not already over-expressing β-catenin and thus cyclin D1.

Here we show that the in all of the $Apc^{Min/+}$ lesions *in vivo* Cyclin D1 (like β-catenin) is over-expressed (figure 9.4). Although this is indicative of β-catenin driving transcription of cyclin D1 its is not definitive proof (see discussion).

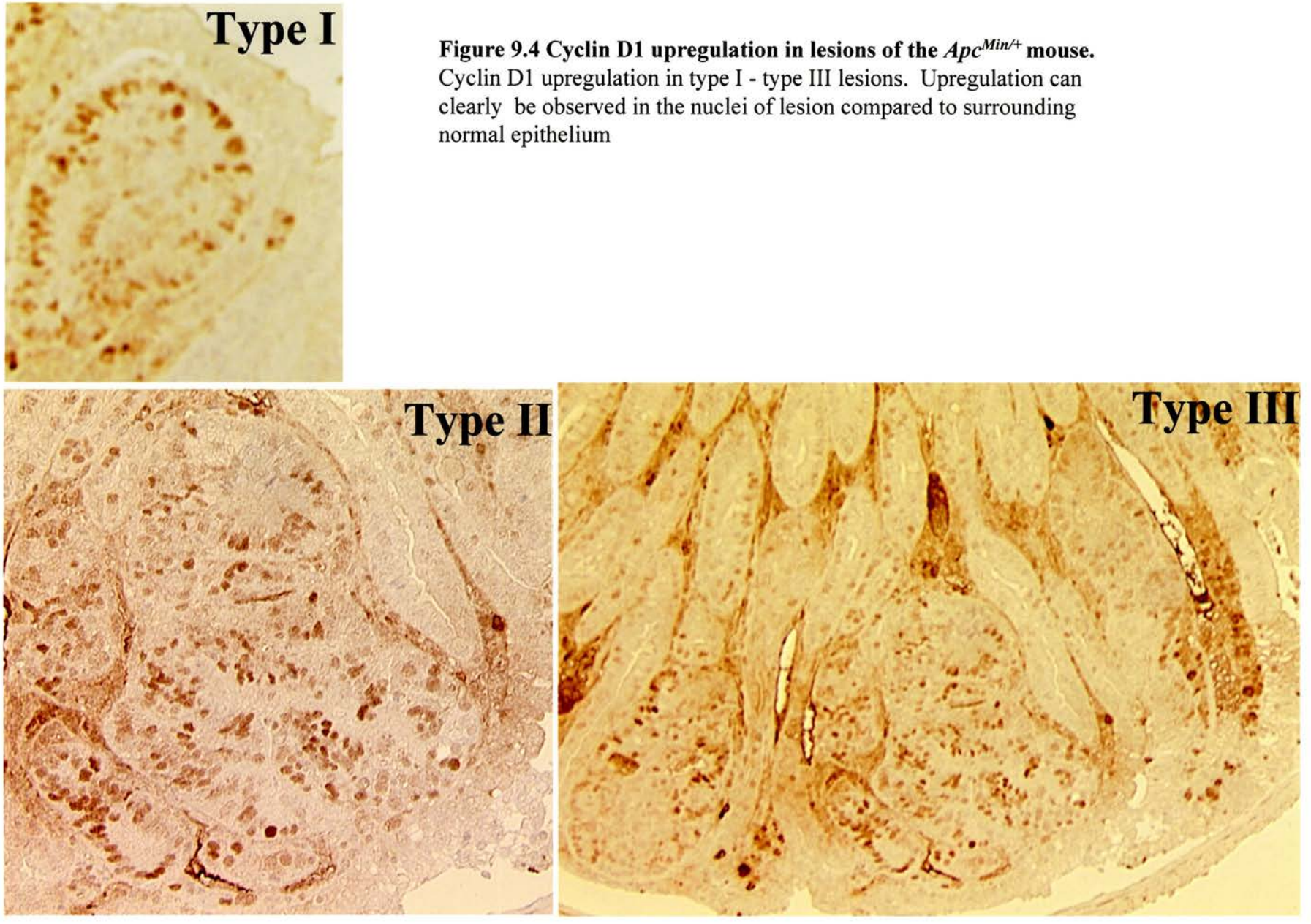


Figure 9.4 Cyclin D1 upregulation in lesions of the *Apc*$^{Min/+}$ mouse. Cyclin D1 upregulation in type I - type III lesions. Upregulation can clearly be observed in the nuclei of lesion compared to surrounding normal epithelium

9.1.4 Spontaneous apoptosis in $Apc^{Min+/-}$ and ($Apc^{Min+/-}$, $Msh2^{-/-}$) lesions

In the $Apc^{Min/+}$ mouse, the incidence of adenocarcinoma (type V) was very rare as were large adenomas (type IV) which were predominantly located in the large intestine. This situation is exacerbated in the ($Apc^{Min/+}$, $Msh2^{-/-}$) mice which develops many small lesions (over a 100) (Reitmair *et al.*, 1996A). These probably cause ill-health and anaemia in the mouse before lesions can progress to type (IV) and (V) lesions. Therefore apoptosis was scored in the small intestine in the type I (single lesions) to type III (adenomas) lesions.

Figure 9.5. Apoptosis in lesions from $Apc^{Min/+}$and ($Apc^{Min/+}$, $Msh2^{-/-}$) mice. Percentage of cells undergoing apoptosis in mock treated mice. Each bar represents at least 3 mice and error bars represent SD. For each mouse at least 50 normal ½ crypts were scored, 10 type I (single crypt) lesions, 10 type II lesions and 5 adenomas. Blue bars, $Apc^{Min/+}$ mice; yellow bars, ($Apc^{Min/+}$, $Msh2^{-/-}$) mice.

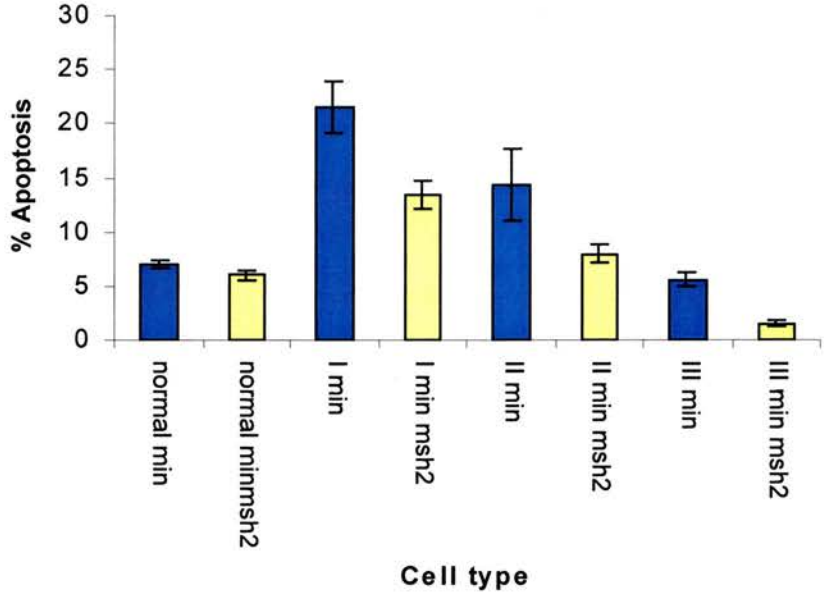
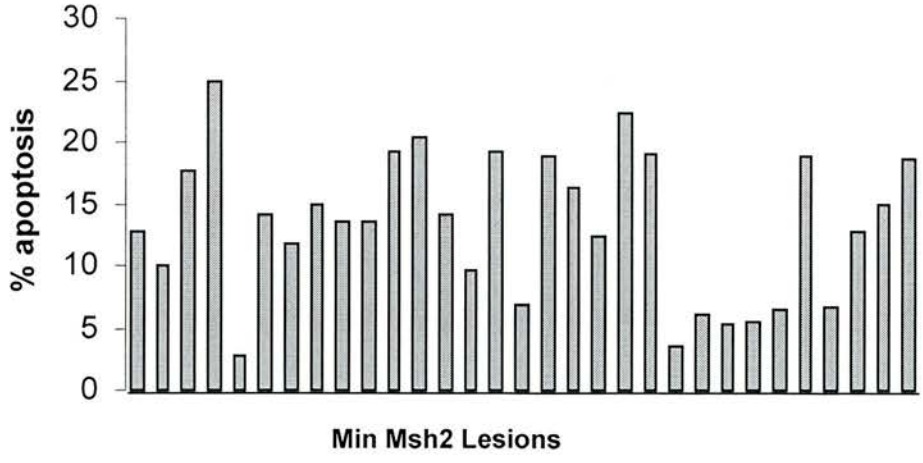


Figure 9.5 shows there is a reduction in apoptosis during intestinal neoplasia in both the $Apc^{Min/+}$and ($Apc^{Min/+}$, $Msh2^{-/-}$) mice. However it appears to be much

more complex than a simple reduction in apoptosis with progression of tumourigensis which was reported in human colorectal neoplasia (Bedi *et al.*, 1995). Compared to normal epithelium, there was a large increase in the levels of apoptosis within the smallest type I lesion for lesions arising in both $Apc^{Min/+}$ and ($Apc^{Min/+}$, $Msh2^{-/-}$) mice (Mann Whitney p=0.04). The type II complex lesions had intermediate levels of apoptosis between the type I lesions and the type III adenomas. The type III adenomas had the lowest levels of apoptosis.

The ($Apc^{Min/+}$, $Msh2^{-/-}$) mice have lower levels of apoptosis than the $Apc^{Min/+}$ mice in the single lesions (p=0.04 Mann Whitney). When the distribution of apoptosis was examined in the ($Apc^{Min/+}$, $Msh2^{-/-}$) mice on a per lesion basis there was the occasional type I lesion with a low level of apoptosis (Figure 9.6). It is possible that these lesions with the low levels of apoptosis are the ones that give rise to the larger type II and III lesions, with the lower levels of apoptosis

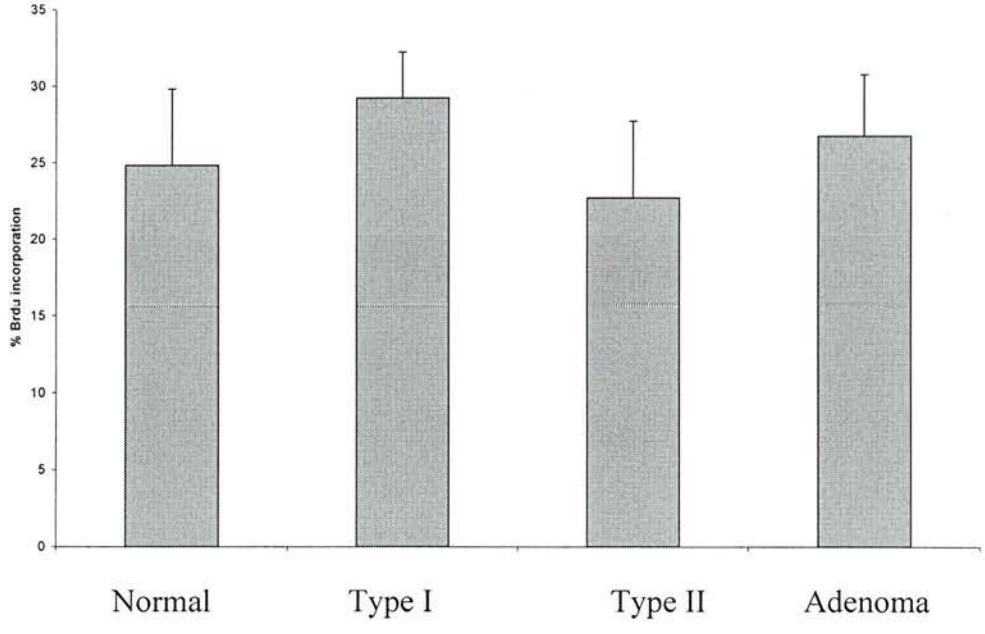
Figure 9.6. Profile of Apoptosis in the untreated type I lesions from ($Apc^{Min/+}$, $Msh2^{-/-}$) mice. Percent of cells undergoing apoptosis in all type I lesions scored in the ($Apc^{Min/+}$, $Msh2^{-/-}$) mice. Each point represents apoptosis in one lesion.



9.1.5 Proliferation in lesions from the *Apc*$^{Min/+}$ mice.

Previously Oshima *et al.,* (1997) have shown that there were no obvious difference in cellular proliferation between adenomas and normal epithelium in *Apc*$^{Min/+}$ mice. As there were clear differences in the level of apoptosis between lesion types, the levels of proliferation were also investigated. This was done by injecting Bromodeoxyuridine (BrdU) into the mice two hours prior to culling. BrdU incorporates into the DNA at S Phase and thus gives an estimation of the number of cells cycling into S phase.

Figure 9.7. No difference in BrdU incorporation within the lesions types of the *Apc*$^{Min/+}$ mice. Percentage of live cells labelled with BrdU, two hours after BrdU pulse. Each bar represents at least 3 mice and error bars represent SD. For each mouse at least 50 normal ½ crypts were scored, 10 single crypt lesions, 10 type II lesions and 5 adenomas.



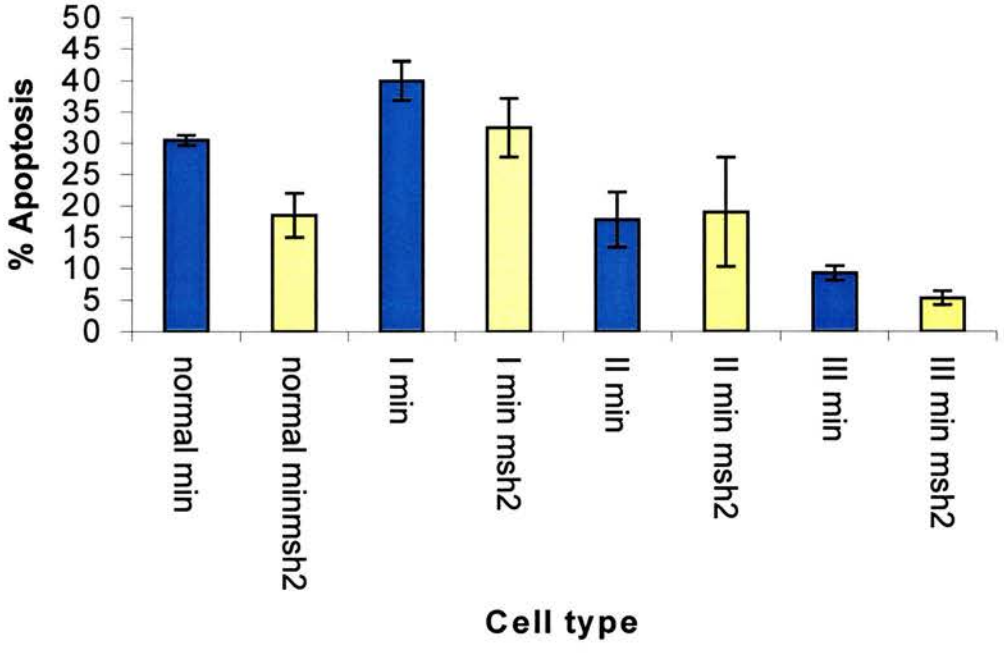
Despite very different levels of apoptosis in the different lesion types there were no significant differences in Brdu incorporation (p=<0.20 for all groups, Mann Whitney). Therefore the increases in the levels of apoptosis observed in type I

lesions is not being counteracted by an increase in proliferation. This consolidates the view that loss or reduction of apoptosis is important for tumour progression.

9.1.6 Apoptosis in tumours after temozolomide treatment

To investigate whether the gene depenedency of the apoptotic response is retained in the adenomas, levels of apoptosis 6 hours following temozolomide exposure were investigated in the lesions of $Apc^{Min/+}$ and ($Apc^{Min/+}$, $Msh2^{-/-}$) mice.

Figure 9.8 Apoptosis in lesions from $Apc^{Min/+}$ and ($Apc^{Min/+}$, $Msh2^{-/-}$) mice 6 hours following 100mg/kg temozolomide treatment. Percentage of cells undergoing apoptosis in temozolomide treated mice. Each bar represents at least 3 mice and error bars represent SD. For each mouse at least 50 normal ½ crypts were scored, 10 type I (single crypt) lesions, 10 type II lesions and 5 adenomas. Grey bars, $Apc^{Min/+}$ mice; open bars, ($Apc^{Min/+}$, $Msh2^{-/-}$) mice.



In chapter 4, apoptosis post temozolomide treatment was shown to be *Msh*2-dependent in normal mucosa. In both normal epithelium and adenomatous tissue (type III lesion) a reduced apoptotic response was seen following temzolomide treatment in the ($Apc^{Min/+}$, $Msh2^{-/-}$) mice compared to the $Apc^{Min/+}$ mice (Mann Whitney p=0.04). This highlights that gene dependency of the apoptotic response is retained in tumours.

Interestingly, in the small lesions: type I and II, no significant *Msh2* dependent apoptosis was observed (p=<0.2, Mann Whitney). This argues that these small lesions (which already have high levels of apoptosis) are primed to undergo apoptosis and thus are exquisitely sensitive to DNA damage.

9.2 Discussion

Using transgenic mice mutant for the tumour suppressor gene $Apc^{Min/+}$ and the DNA mismatch repair gene *Msh2*, the pattern of β-catenin expression in neoplasms within the murine intestine has been characterised. It has been shown that high levels of β-catenin are present in the majority of intestinal lesions, and that dysregulation of β-catenin is associated with loss of the remaining wild type copy of *Apc*, presumably as a direct consequence of perturbation of the Wnt pathway. Areas showing high levels of β-catenin included those composed of heterogeneous or single dysplastic crypts in the intestine. Histological atypia was always observed within these foci in the intestine. Taken together, these findings show that dysregulated β-catenin expression is an extremely efficient marker of early neoplastic change in the murine intestine. In fact, it is relatively surprising that in the intestine, there was no incidences of normal crypts showing over-expression of β-catenin, especially as it is argued that the number of adenomas in the $Apc^{Min/+}$ mice is fixed *in utero* (Shoemaker *et al.,* 1994, De Wind *et al.,* 1998). This argues that the time between β-catenin over expression in a 'normal cell' that has lost *Apc* and the morphological appearance of a dysplatic cell is relatively short.

Although β-catenin dysregulation is shown to be a common event here, it is not absolutely associated with early neoplastic change. Thus, type II lesions were identified arising in both $Msh2^{-/-}$ and ($Apc^{Min/+}$, $Msh2^{-/-}$) mice which showed normal levels and distribution of β-catenin. These findings show that dysregulated β-catenin is not an obligate event in early lesion formation, and furthermore that *Msh2* deficiency predisposes to such apparent β-catenin-independent events. One explanation for this is that *Msh-2* deficiency may predispose to dysplasia through mutations in other components of the Wnt signalling pathway which do not affect β-catenin levels. In MMR deficient colorectal tumours mutations have been found in axin and TCF4 (Duval *et al.,* 1999,). However axin mutations would still lead to upregulation of β-catenin. In MMR deficient tumours, frequent frameshifts have been shown in TCF4 in a A(9) tract of the TCF4 gene. These

produce truncated proteins that lack the c-terminal portion of TCF4 which has been implicated to be important in repression of β-catenin/TCF4 transcription. Once again, this probably only becomes important once β-catenin is over-expressed. This is highlighted by the synergy between mutations in $Apc^{Min/+}$ and $Tcf1^{-/-}$ null mice (mentioned in 1.3.1). $Tcf1^{-/-}$ mice very rarely develop intestinal and mammary adenomas, however when they are crossed to $Apc^{Min/+}$ mice, there is both a much earlier onset and increased frequency of these tumours (Roose *et al.,* 1999).

Another possible explanation for the lack of upregulation of β-catenin in a subset of $Msh2^{-/-}$ and ($Apc^{Min+/-}$, $Msh2^{-/-}$) lesions is that mutations in other pathways can produce these dysplatic lesions. MMR deficiency increases the likelihood of mutations occurring within other critical loci. One potential target is the TGFβ pathway, which is important for signalling cellular proliferation and apoptosis. Loss of DPC4/Smad4, a central mediator of TGFβ signalling, has been shown to occur in a number of colorectal cancers (Vogelstein and Kinzler 1996, Miyaki *et al.,* 1999). More importantly in HNPCC, mutations in the TGFβ RII are relatively frequent (Akiyama *et al.,* 1997). Indeed one HNPCC family had no obvious MMR mutation yet had a germline TGFβ RII mutation (Lu *et al.,* 1998). This indicates that dysregulation of this pathway could potentially lead to the dysplatic crypts. However all small adenomas (type III) were characterised by increased β-catenin, suggesting that adenoma formation is absolutely associated with changes which modulate β-catenin levels. This concords with the hypothesis that *Apc* is the key intestinal 'gatekeeper' in prevention of malignancy (Vogelstein and Kinzler 1996)

Taken together, these results show that β-catenin dysregulation is associated with the early steps in the development of neoplasia. These findings demonstrate that altered expression of β-catenin is a key marker of *Apc* dysregulation. However, it is also shown that β-catenin dysregulation is not an obligate step in the generation

of intestinal lesions in a *Msh2* deficient background, and therefore that other mechanisms can underlie such early neoplastic change.

The increased levels of cyclin D1 observed in the lesions/adenomas in conjunction with high expression of β-catenin suggests that the major reason for loss of *Apc* in these adenomas is for inappropriate Wnt signalling. Two studies have previously shown that cyclin D1 is a downstream target of Wnt signalling and one study has shown that cyclin D1 is overexpressed in adenomas (Zhang *et al.,* 1997). This is the first *in vivo* confirmation that in murine intestinal neoplasia that expression of both β-catenin and cyclin D1 are dysregulated. However this is purely correlative. One argument against this direct mechanism is that the expression of cyclin D1 may merely be an indicator of the increased proliferation in the dysplatic tissue rather than any true reflection of Wnt signalling. However as is shown here and by Oshima *et al.,* (1997), there is no difference in cellular proliferation (as measured by BrdU incorparation) between normal intestinal epithelium and adenomatous tissue in the $Apc^{Min/+}$ mouse. This indicates that the cyclin D1 over-expression observed in the lesions is not simply a reflection of increased proliferation in the intestinal lesions and instead more likely represents dysregulated Wnt signalling.

From other systems, other potential targets of Wnt signalling are *C-myc*, *AP-1*, *Tcf4* etc (see 1.4.1). Therefore one would predict that inappropriate Wnt signalling would cause increased proliferation and/or death. The paradigm for this is the activity of c-myc which has been shown to induce both proliferation and apoptosis depending on the cellular context (Evan *et al.,* 1992). Previously Bedi *et al.,* (1995) showed that there is a relative inhibition of apoptosis when normal colorectal epithelium is compared to adenoma/adeno-carcinomas. The detailed analysis of apoptosis performed here shows that this is in fact an over-simplification and there is a specific induction of apoptosis in the very small dysplatic lesions compared to normal epithelium. As the tumour progresses to adenoma, there is a reduction of apoptosis compared to both normal mucosa and the dysplatic crypts (type I and II). This is highly indicative that loss of apoptosis

is important for tumour progression. This also concurs with our understanding of oncogenes such as *c-myc* and β-catenin and progression in murine intestinal neoplasia. Therefore in the small lesions, high abberant expression of β-catenin, *cyclin D1* and possibly *c-myc* induce apoptosis rather than proliferation as the cells may still have an intact apoptotic programme. However selection against apoptosis would select for those cells that lose their apoptotic response and lead to lesions with lower levels of apoptosis. Again this correlates with the molecular changes seen in intestinal neoplasia. For example Oshima *et al.,* (1997) showed that the Tgfβ1 receptor and Tgβ receptor II were expressed in microadenomas (equivalent to lesion I and II) and not in adenomas (type III). This is important as loss of Tgfβ signalling is thought to cause a reduction in apoptosis.

Following damage, *p53* is widely acknowledged as a central mediator of the apoptotic response. It seems unlikely that loss of *p53* plays any role in the loss of these basal levels of apoptosis from the type I lesion to the type III lesions. The reason for this is that *P53* is generally lost post adenoma stage when apoptosis is already reduced. Fazeli *et al.,* (1997) showed that there were no differences in the levels of apoptosis between adenoma and adeno-carcinomas. Similarly *p53* dependent apoptosis in the tumours was retained between $Apc^{Min/+}$ tumours and ($Apc^{Min/+}$, $p53^{-/-}$) tumours following gamma irradiation. This indicates that in these $Apc^{Min/+}$ adenomas there is functional *p53*. Likewise apoptosis following temozolomide at 6 hours is *p53* dependent so if *p53* was absent no apoptosis at this timepoint would be seen. Furthermore, the lack of an obvious increase in tumourigenesis in ($Apc^{Min/+}$, $p53^{-/-}$) mice compared to the $Apc^{Min/+}$ mice argues that it is unlikely the spontaneous high levels of apoptosis observed in type I lesions is dependent on p53. Therefore it would be interesting to investigate whether there are increased levels of apoptosis in ($Apc^{Min/+}$, $p53^{-/-}$) type I and II lesions.

When apoptosis was induced following temozolomide, in both the adenomatous and the morphologically normal epithelium, there was *Msh2*-dependent apoptosis. However, in the type I and type II lesions there was no significant *Msh2*-dependent apoptosis. This is even more apparent if one takes into account that

basal levels of apoptosis were lower in type 1 and II lesion arising in the ($Apc^{Min/+}$, $Msh2^{-/-}$) mice. Thus, it could be argued that there would be selection for loss of *Msh2* in untreated type I and II lesions and in intestinal adenomas following treatment with alkylating agents. In this instance (unlike chapter 4) this fits with the Tomlinson and Bodmer (1999) argument that loss of MMR delivers a selective advantage through loss of apoptosis rather than a mutator phenotype.

This would also suggest that MMR deficient adenomas would not respond as well to alkylating agents as MMR proficient tumours. However this is assuming that this induced apoptosis is an important critea for chemotherapy to work. In chapter 5, it was shown that despite having a completely abrogated apoptotic response to NMNU, $p53^{-/-}$ mice unlike $Msh2^{-/-}$ mice did not have an increased clonogenic survival in the small intestinal epithelium. From the apoptotic data alone, one would have predicted that *Msh2* and *p53* deficient tumours would not respond to alkylating agents, though from the clonogenic data it would have been predicted that *Msh2* deficient tumours would not respond whilst *p53* deficient tumours would. Therefore further experiments are required to see which is the better indicator of tumour prognosis, apoptosis or clonogenic survival.

Another interesting point raised from the induction of apoptosis in tumours is that normal intestinal epithelium appeared more sensitive than the adenomatous tissue. This raises questions on the widely held view that chemotherapy works through the specific induction of apoptosis in the tumours. Clearly some apoptosis is occurring in the tumours but this is much less than that occurring in the normal intestinal epithelium. The level of apoptosis in normal tissue was 30% in normal tissue compared to 8% in the adenomas. Given that in humans the majority of colorectal tumours would have lost P53, one would predict that this situation would be exacerbated. Fazeli *et al.,* (1997) showed that basal levels of apoptosis in $Apc^{Min/+}$ adenomas were 2-3% apoptosis compared to approximately 17% after 6 hours after 8 GY gamma irradiation. However there was no induction of apoptosis at this timepoint in the ($Apc^{Min/+}$, $p53^{-/-}$) lesions following gamma irradiation. Therefore as normal colorectal epithelium in humans would have

retained *P53*, one would predict levels of apoptosis would be far greater in this normal epithelium following chemotherapy than in colorectal tumours. This makes it very hard to reconcile that chemotherapy is working via the specific induction of apoptosis in the neoplastic tissue. In fact it almost appears to be working in an opposite manner!

In this study temozolomide has been used which is not a treatment of colorectal cancer and so could explain the lack of a specific induction of apoptosis in the tumours. However the Fazeli *et al.,* (1997) study used gamma irradiation which is still one of the principle agents for treating colorectal cancer. Therefore, as was stated in chapter 5, if treatment was being tailored to p53 status, no colorectal cancer would be treated with gamma irradiation. These studies have only looked at apoptosis at one timepoint, thus it possible at later timepoints there could be elevated levels of apoptosis in the adenomas. Therefore it would be interesting to score apoptosis at later timepoints following temozolomide treatment in lesions from $Apc^{Min/+}$ and ($Apc^{Min+/-}$, $Msh2^{-/-}$) mice

Some care should be always be exercised in interpreting the relevance of murine small intestinal epithelium to human colorectal cancer. However these studies highlight that apoptosis in intestinal neoplasia is complex and that assessing whether chemotherapy will work based on whether it induces apoptosis is a gross over-simplification. It also highlights that we are far from understanding the mechanism of how many chemotherapeutics works. For example, cisplatin cures approximately 90% testicular cancers though why it is so successful is still unknown. Other drugs produce similar lesions, levels of apoptosis and arrest profiles, yet they do not work as effectively. Therefore although there is now the technology to genetically profile tumours for many different genes (e.g. by microarray) as yet informed decisions on whether certain chemotherapeutics will or will not work from the genetic background of the tumour alone cannot be made.

Instead as the ability to specifically inhibit pathways in tumours with less toxic drugs/gene therapies grows, hopefully this may offer new treatments. Examples of these include the inhibition of *Cox-2* (cyclo-oxygenase 2) in colorectal tumours by selective *Cox-2* inhibitors (which I will discuss in the next chapter) and deletion of *P53* null cells using ONYX015 adenovirus which replicates in *P53* deficient cells. None of these rely on potentially false assumptions concerning apoptosis, proliferation and resistance.

Chapter 10: Suppression of intestinal and mammary neoplasia by lifetime administration of aspirin in *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$, *Msh2*$^{-/-}$ mice.

10.0 Introduction

During the course of this thesis the difficulties of translating apoptosis to long term measures of survival have been discussed. NSAIDS (Non-Steroidal Anti Inflammatory Drugs) such as aspirin and sulindac have also been shown to induce apoptosis (Chen *et al.,* 1999, Stark *et al.,* 2001). Unlike the cytotoxic agents (temozolomide, cisplatin and nitrogen mustard) used, these NSAIDS do not elicit DNA damage and thus are not as carcinogenic or toxic to patients. This allows potential for chemoprevention with these agents in addition to chemotherapy. Importantly, NSAIDS have been shown to down regulate pathways that are dysregulated in colorectal cancer, thus raising the hope of treatment targeting tumours specifically. These targets down regulated by NSAIDS include β-catenin (shown to be upregulated in the last chapter), PPAR delta (a target of Wnt signalling), and Cox2 (upregulated in adenomas in the $Apc^{Min/+}$ mouse). In addition Ruschoff *et al.,* (1999) showed that aspirin and sulindac may delete cells that exhibit MSI, again which should preferentially kill tumour cells. Therefore given that there was still confusion over the role for aspirin in suppression of intestinal tumourigenesis in the mouse, it seemed an ideal agent to test whether it could suppress intestinal neoplasia in $Apc^{Min/+}$ and ($Apc^{Min/+}$, $Msh2^{-/-}$) mice

Substantial epidemiological evidence shows that treatment with NSAIDS reduces the risk of developing colorectal cancer. The most commonly used NSAID has been aspirin, which has been reported to reduce the risk of colon cancer by up to 40%. Clinical studies using the NSAID sulindac have also reported reduced polyp counts in FAP patients (Thun *et al.,* 1991, Giardiello *et al.,* 1993). The precise mechanism of NSAID action remains unclear, although the suppression of cyclooxygenase 2 (Cox2) is thought to be of pivotal importance. Significantly, Cox2 is over-expressed in approximately 85% of human colorectal adeno-carcinomas and adenomas from the $Apc^{Min/+}$ mouse (Eberhart *et al.,* 1994, Williams *et al.,* 1996, Williams *et al.,* 1999). The definitive study highlighting the relevance of Cox2 to tumourigenesis showed that a Cox2 deficient background markedly suppressed intestinal neoplasia in mice carrying the $Apc^{\delta 716}$ allele (Oshima *et al.,* 1996).

The biological activities of aspirin and sulindac are not restricted to suppression of the Cox2 pathway. This is exemplified by the fact that both sulindac metabolites (sulindac sulfide and sulindac sulfone) have been shown to inhibit animal models of tumourigenesis, even though only sulindac sulphide inhibits COX2 (Piazza *et al.,* 1997). However it must be added that of the two sulindac metabolites sulindac sulfide is the most effective at tumour suppression.

Aspirin, sulindac and even the selective Cox2-inhibitor celecoxib have been shown to induce apoptosis in a Cox2 independent fashion (Williams *et al.,* 2000). Both aspirin and sulindac down regulate beta-catenin and beta-catenin/ TCF4 mediated transcription (Mamhood *et al.,* 1998a, 1998b, McEntee *et al.,* 1998, Dihlmann *et al.,* 2001). They have also been shown to specifically reduce the survival of genetically unstable (MSI+) MMR deficient colorectal cancer cell lines (Ruschoff *et al.,* 1998), raising the possibility that aspirin may also suppress malignancy in HNPCC families characterised by mutations in the MMR genes.

Mice constitutively heterozygous for the Apc^{Min} allele have been used to determine the ability of the NSAIDs to suppress intestinal malignancy. However with respect to aspirin, these studies have produced contrasting results. Virtually every study (e.g. Beazer-Barclay *et al.,* 1996, Mamhoud *et al.,* 1998b) has shown that sulindac causes a reduction in the number of the spontaneous malignancies (apart from Oshima *et al.,* 1996) who used a relatively low levels of sulindac). However, of the studies that have investigated spontaneous intestinal malignancy following aspirin treatment, only two have shown suppression of malignancy in the $Apc^{Min/+}$ mouse. Two further studies failed to show suppression in either the $Apc^{Min/+}$ mouse or the Apc^{1638N} mouse (Mamhoud *et al.,* 1998a, Barnes and Lee 1998, Williamson *et al.,* 1999, Chiu *et al.,* 2000). The basis for these discrepancies may lie within differences in the aspirin regime used. Shoemaker *et al.,* (1995) and Reitmair *et al.,* (1996) have argued that the majority of adenomas are fixed either *in utero* or perinatally just after birth.

Here to directly test this, the period of aspirin exposure was increased to include the entire period from the point of conception onwards. Furthermore whether this regime could modify the development of MMR associated neoplasia was also tested by investigating the course of neoplasia in cohorts of *Msh2*$^{-/-}$ and (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) mice.

10.1 Results

10.1.1 Aspirin does not suppress intestinal neoplasia when administered at weaning

Wild type and $Apc^{Min/+}$ mice were placed on aspirin containing diets (either 200mg/kg and 400mg/kg) at weaning. Mice exposed to the aspirin diet gained weight normally when compared to mice on the control diet and no ulceration or intestinal pathology such as perforation of the intestine was observed in any of the wild type mice treated with aspirin. Two independent analyses were performed.

First, cohorts of mice either wild type or heterozygous for the Apc^{Min} allele were killed at 150 days of age and the adenoma burden assessed on whole mount preparations of the entire small intestine. No adenomas were seen in wild type mice. Adenoma burden was as follows: for mock treated $Apc^{Min/+}$ mice (n=5) 9.8 (+/- 6.3); mice treated with 200mg/kg (n=7) 9.7 (+/- 5.88) ; for mice treated with 400mg/kg of aspirin (n=5) 8.2 (+/- 6.05). There was no difference in tumour burden at either 200mg/kg (p=1.00) or at 400mg/kg (p=0.75 Mann Whitney) compared to mock treated mice, indicating that aspirin exposure was not modifying the $Apc^{Min/+}$ phenotype

Second, cohorts of mice were permitted to age until they developed obvious symptoms of intestinal neoplasia, usually bleeding from the anus or anaemia scored through whitening of the feet.

Fig 10.1 Kaplan Meier plot of survival of *Apc*$^{Min/+}$ exposed to control or aspirin containing diet. Black line, mock treated mice (n=15); red line, 200mg/kg diet (n= 12); blue line, 400mg/kg of diet (n=10). There was no significant difference in the survival curves of either the mice treated with 200mg/kg (Log Rank p= 0.96) and 400mg/kg (Log rank p=0.30).

Green line, *Apc*$^{Min/+}$ mice exposed to aspirin containing diet aspirin from point of conception. These mice (n=16) showed a significant increase in survival (Log rank, p= 0.0004) compared to mock treated and aspirin treated mice at weaning.

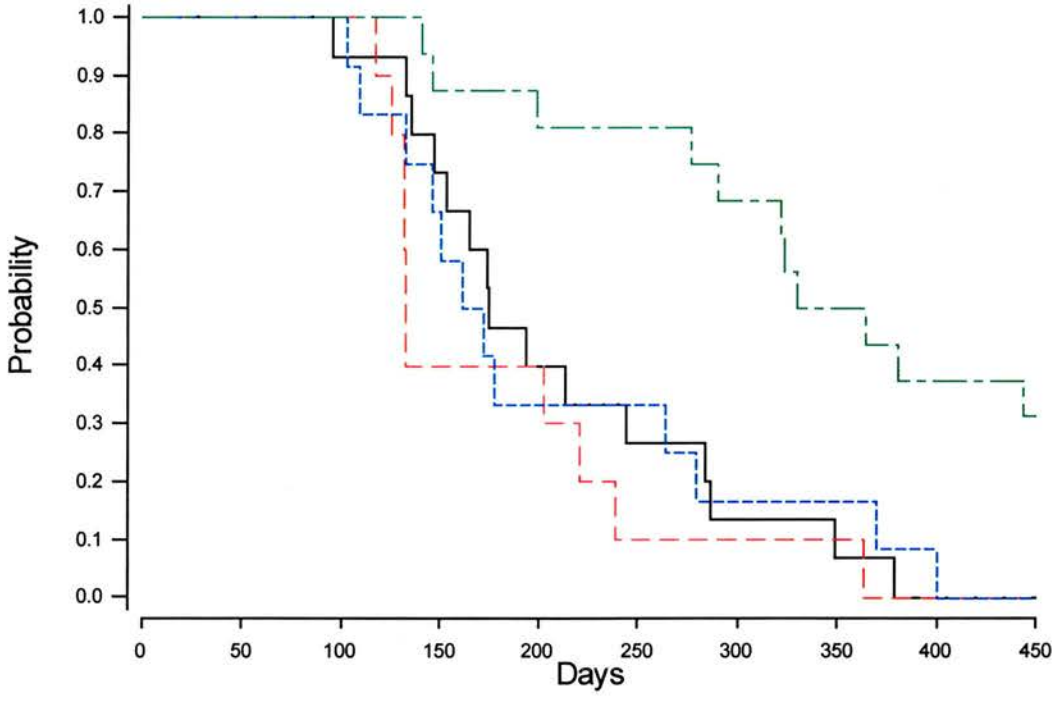


Figure 10.1 shows a Kaplan Meier plot reflecting survival over a 400 day period. Exposure to aspirin at either 200 mg/kg or 400 mg/kg did not alter the survival profile, again indicating that exposure to aspirin did not modify the *Apc*^{Min} phenotype.

10.1.2 Aspirin suppresses intestinal neoplasia when administered *in utero*

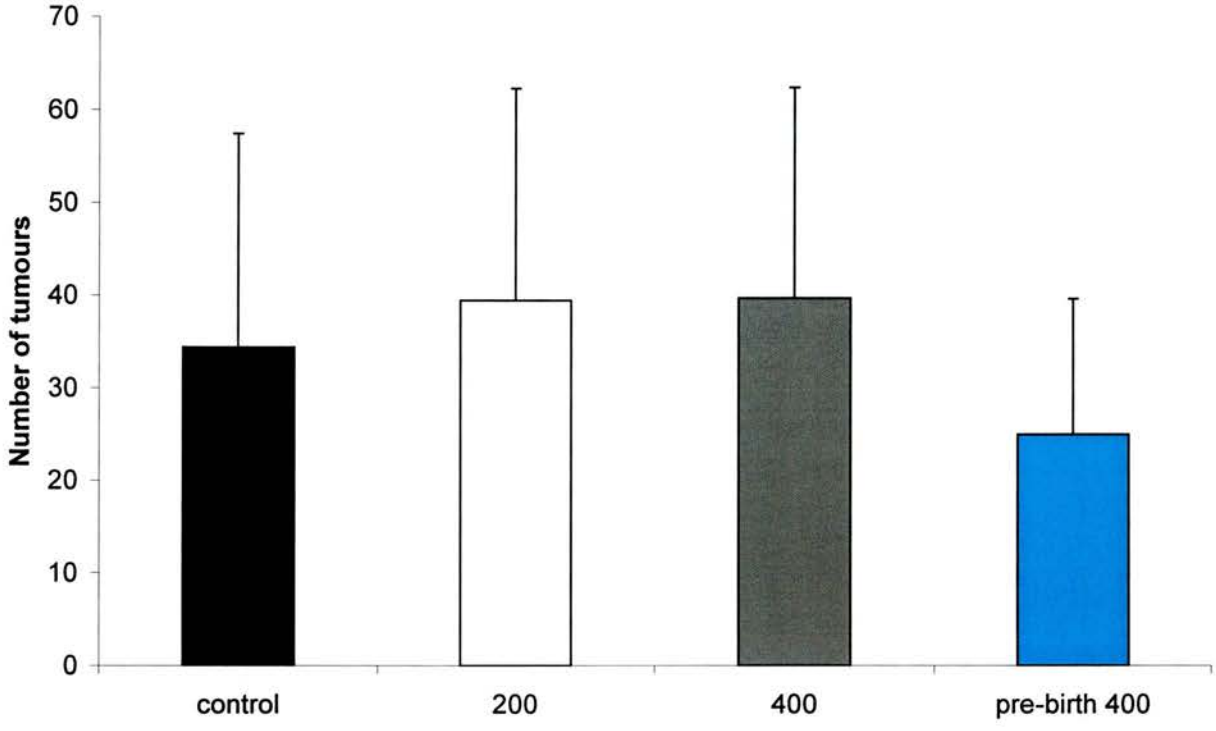
As Shoemaker *et al.*, (1995) and Reitmair *et al.*, (1996) have argued that the majority of adenomas are fixed before 6 days of age, the effect of aspirin exposure throughout embryogenesis and weaning was investigated. Cohorts of wild type and $Apc^{Min/+}$ heterozygotes were derived from parents placed on aspirin containing diet prior to conception. Dietary exposure to aspirin was maintained throughout and beyond weaning. First it was investigated whether *in utero* exposure resulted in increased embryonic lethality of $Apc^{Min/+}$ heterozygotes, as has been reported for the Cox 1 and Cox2 inhibitor piroxicam (Jacoby *et al.*, 2000). Analysis of offspring showed this not to be the case, as there was no reduction in the number of $Apc^{Min/+}$ heterozygotes in progeny from Aspirin exposed parents (p=0.2, Chi-squared test).

Figure 10.2: Numbers of mice from $Apc^{Min/+}$ crosses to wild type mice.

	$Apc^{Min/+}$	Wild type
Untreated	99	100
In Utero exposure	50	63

The survival profiles of each cohort were then determined (figure 10.1), they showed a significant increase in survival in $Apc^{Min/+}$ heterozygotes exposed to aspirin from conception onwards (p=0.0004, Log Rank test). This effect was sufficient to completely prevent the development of symptoms associated with intestinal neoplasia in 5/16 Apc^{Min} at 500 days of age. Analysis of tumour burden and distribution in those mice that developed intestinal tumours showed no obvious differences (figure 10.3).

Figure 10.3: Tumour burden of *Apc*$^{Min/+}$ mice. Tumour burden at point of sacrifice as determined by onset of overt symptoms of intestinal tumourigenesis. Black bars, mock treated mice; open bars, mice treated with 200mg/kg; grey bars, mice treated with 400mg/kg of aspirin at weaning; and blue bars, mice treated with 400mg/kg of aspirin from point of conception.



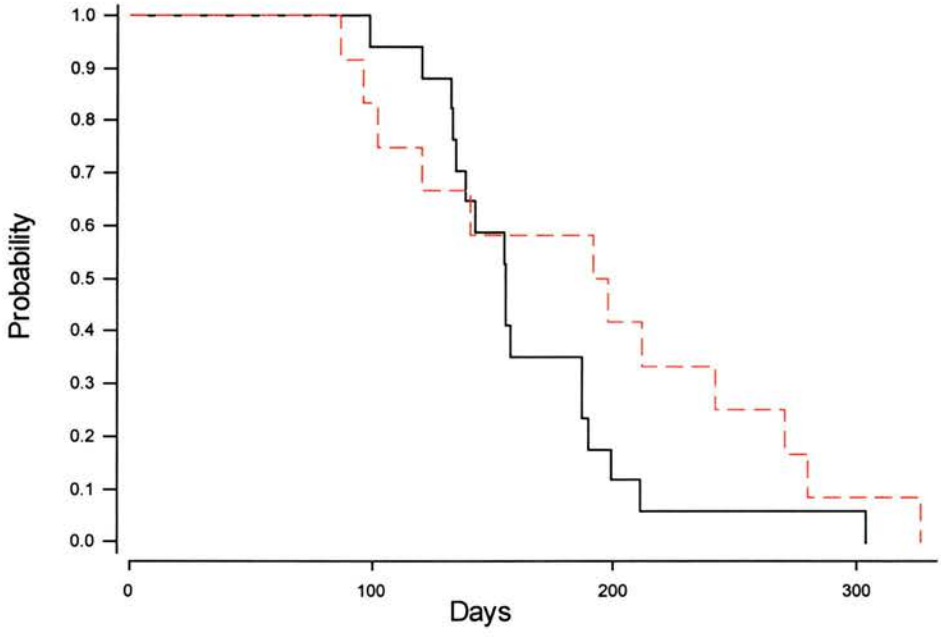
There was no significant difference in the number of tumours at sacrifice between mock exposed mice (n=15) and mice exposed to 400mg/kg from conception (Mann Whitney p=0.613) (n=9). Tumour burden of mice aged over 500 days old were excluded from this analysis as these mice were killed when symptomatic of lymphoma and not intestinal neoplasia.

10.1.3 Aspirin weakly suppresses neoplasia though not mutation in Msh2$^{-/-}$ mice

Having established an effective protocol for aspirin exposure in the murine model of FAP, it was determined if this approach could modulate the phenotype of the murine model of HNPCC. A significant subset of human intestinal tumours are characterised by mutations in the MMR pathway, and all murine models of MMR deficiency show increased predisposition to neoplasia.

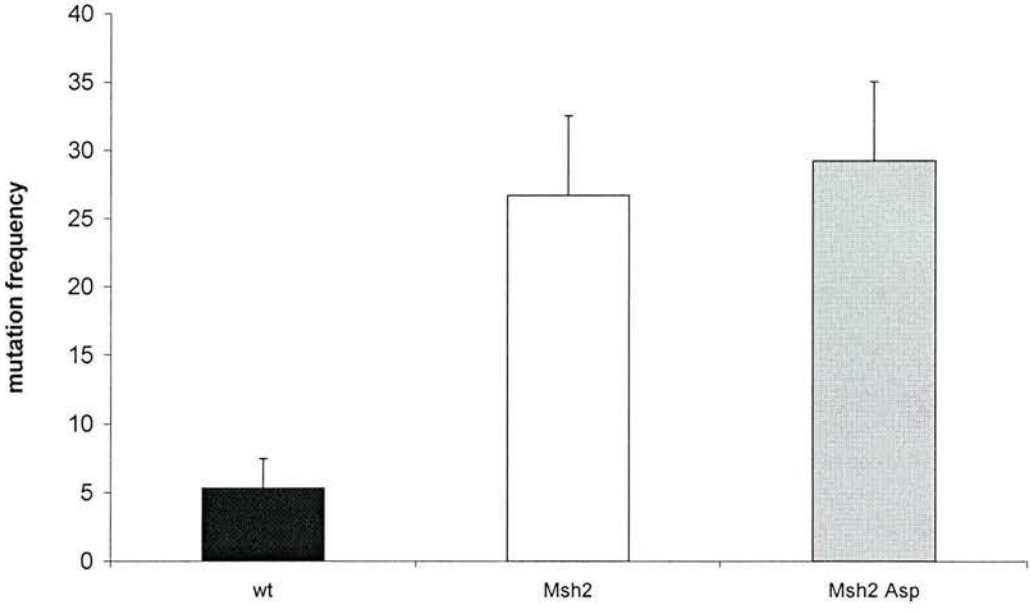
Cohorts of *Msh2*$^{-/-}$ mice were either exposed to aspirin or were fed control diet from conception and Kaplan Meier survival curves generated (figure 2A). All eighteen animals on the control diet were killed following the development of lymphoma, although two animals also had co-existent intestinal malignancy. All *Msh2*$^{-/-}$ mice exposed to aspirin also developed lymphoma, however there was a small increase in survival compared to controls (p=0.05, Log Rank). This slight shift in survival may reflect weak suppression of either intestinal malignancy or lymphomagenesis. This latter possibility is consistent with the one report that showed aspirin exposure can result in the deletion of MSI unstable cells in culture (Ruschoff *et al.,* 1998).

Figure 10.4 Kaplan Meier plot of survival of *Msh2*$^{-/-}$ mice exposed to control or aspirin containing diet from point of conception. Solid black line, mock treated *Msh2*$^{-/-}$ mice (n=18); red hatched line, 400mg/kg treated *Msh2*$^{-/-}$ mice (n=20). *Msh2*$^{-/-}$ mice treated with aspirin showed a weak increase in survival (Log rank p=0.05).



These results prompted the investigation whether aspirin exposure could suppress *in vivo* mutation in a mismatch repair deficient background. Mutation frequency was scored at the dolichos biflorus (*Dlb-1b*) locus in *Msh2* mutant mice continually exposed to either control or aspirin containing diets. Using this assay, we have previously shown that *Msh2* deficient mice have a mutator phenotype at the *Dlb-1b* locus. *Msh2* deficient mice were analysed at 4 months of age following exposure to either control or aspirin containing diet. No difference in mutation frequency was observed at this time point, demonstrating that this regime of aspirin exposure does not modify the mutator phenotype of *Msh2* null epithelium (figure 10.5).

Figure 10.5: *in vivo* mutation frequency per 10000 villi at the *Dlb-1b* locus after aspirin treatment. Mutation frequencies were determined from intestinal wholemounts at 4 months of age. Columns represent mean mutation frequency at the *Dlb-1b* locus. At least 3 mice were used per each column. Error bars represent SD. Black bar, mock treated wild type mice; open bar, mock treated *Msh2*$^{-/-}$ mice; grey bar, *Msh2*$^{-/-}$ mice exposed to 400mg/kg of dietary aspirin from point of conception. There was not a significant reduction in mutation frequency after aspirin treatment (p= 0.77, Mann Whitney)



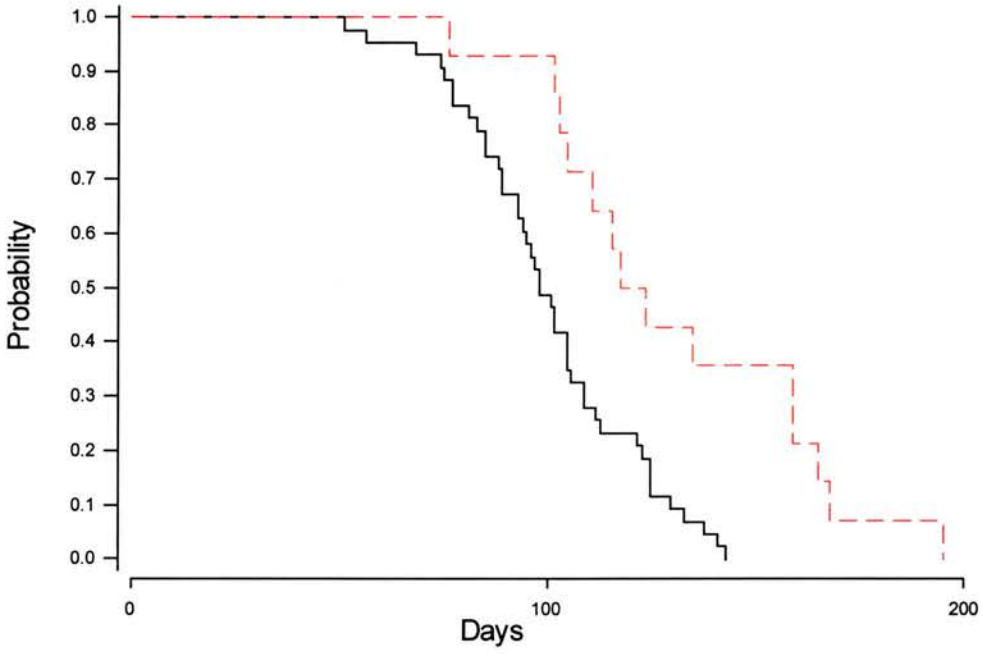
10.1.4. Aspirin supresses intestinal and mammary neoplasia in *Apc*$^{Min/+}$ *Msh2*$^{-/-}$ mice

In human colorectal cancer MMR deficient tumours differ in a number of respects to MMR proficient tumours. For example, MMR deficient tumours express lower levels of Cox2 (Karnes *et al.,* 1998) as well as exhibiting very different patterns of mutation and genomic instability. These differences invoke different mechanisms for MMR

driven neoplasia, raising the possibility that aspirin exposure may have an additional effect in a MMR deficient background, for example through the deletion of cells showing MSI.

The development of lymphoma in *Msh2*$^{-/-}$ mice largely precludes an analysis of the effect of aspirin upon *Msh2* dependent intestinal neoplasia (De Wind *et al.,* 1995). However, *Msh2* deficiency has been shown to greatly accelerate intestinal neoplasia in the *Apc*^{Min} mouse (Reitmair *et al.,* 1996), so permitting an analysis of the effect of aspirin in this context. In addition mammary akanthoma occur in *Apc*$^{Min/+}$ mice (Moser *et al.,* 1993) and this is greatly accelerated in (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) mice, permitting a study of the effect of aspirin on the development of this lesion (see below). Cohorts of (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) mice were generated and exposed to either control diet or aspirin containing diet from conception onwards and Kaplan Meier survival curves generated (figure 10.6). Survival was markedly enhanced following aspirin exposure (Log Rank p=0.0001), although still reduced by comparison to untreated *Apc*^{Min} heterozygotes. Aspirin exposure did not alter either tumour distribution or burden scored at the point of death.

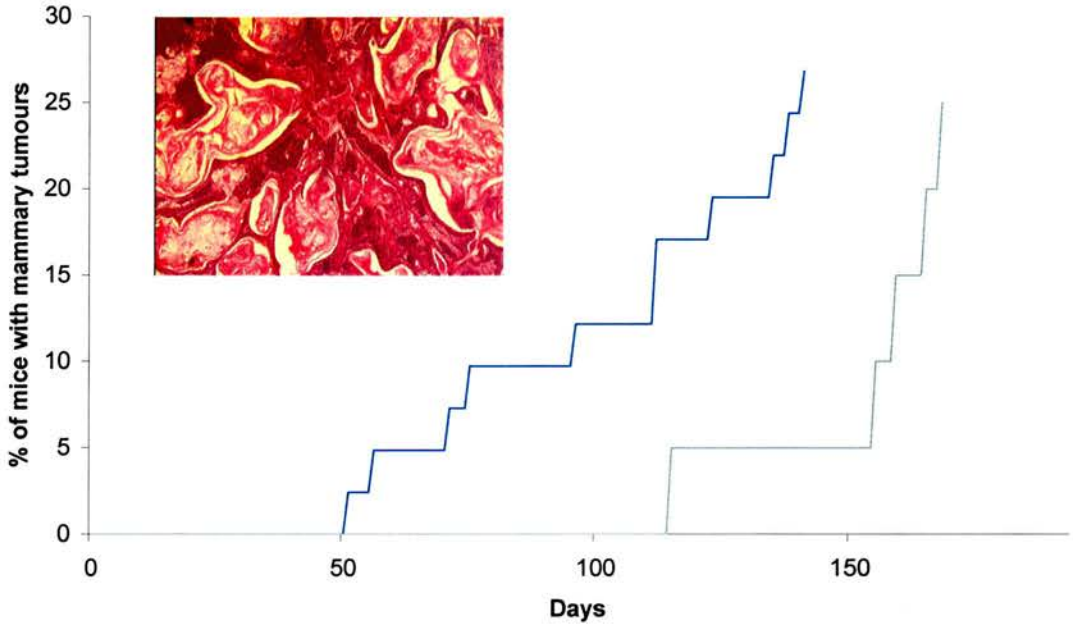
Figure 10.6. Kaplan Meier plot of survival of *Apc*$^{Min/+}$ *Msh2*$^{-/-}$ mice exposed to control or aspirin containing diet from point of conception. Solid black line, mock treated mice (n=43); Red hatched line, mice treated with 400mg/kg from conception(n=17). Aspirin treatment significantly increased survival (Log rank p=0.0001).



In the cohorts maintained on control diet, a single mammary akanthoma was observed in a total of 15 *Apc*$^{Min/+}$ mice, whilst none were detected in 18 *Msh2*$^{-/-}$ mice. Development of this tumour type was enhanced in *Apc*$^{Min/+}$ *Msh2*$^{-/-}$ mice (11 tumours in 41 mice). In the cohorts exposed to aspirin, no mammary akanthomas were observed in 10 *Apc*$^{Min/+}$ mice, whilst a single akanthoma was observed in 21 *Msh2*$^{-/-}$ mice. In the (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) cohort, 5 mammary akanthomas were observed in 20 mice. All of these mice also had intestinal neoplasia. Although this represents a similar prevalence to that observed in the untreated (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) cohort, the

onset of akanthoma was significantly retarded in the aspirin treated mice (figure 10.7)

Figure 10.7. Graph showing delayed incidence of mammary tumourigenesis in the (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) mice. Blue line, mock treated mice, Grey line, mice treated with 400mg/kg of aspirin treatment from conception. Inset picture of mammary akanthoma with large keratin whorls.



10.2 Discussion

Over the past 10 years there has been great excitement at the possibility of chemoprevention by NSAIDS. However, contradictory results have been obtained from 4 different analyses using aspirin in the Apc^{Min} murine models of intestinal neoplasia (Mamhoud *et al.,* 1998a, Barnes and Lee 1998, Williamson *et al.,* 1999, Chiu *et al.,* 2000), where aspirin was only observed to suppress tumorigenesis in 2 studies (Mamhoud *et al.,* 1998a, Barnes and Lee 1998).

A number of different possibilities may underlie the discrepancies between the published studies examining the effect of aspirin on Apc^{Min} associated tumourigenesis. First, as a consequence of differences in the level of aspirin exposure, a possibility which seems unlikely given the similar levels used in each experiment. Second, due to differences in the genetic background of the mice used, either at known modifiers of the $Apc^{Min/+}$ phenotype such as *Mom-1* or at as yet unidentified modifiers (Cormier *et al.,* 2000). In this respect, it is notable that this study differs from previous studies by using mice homozygous for the relatively resistant C57/Bl6 *Mom-1* allele. Third, as a consequence of different husbandry regimes; for example, mice housed in a sterile facility may respond very differently to those in a non-sterile facility.

Comparison of the data presented here with published experiments does not permit which of these possibilities are true, but does serve to underline the relatively fragile nature of aspirin mediated suppression within this model. Here it is shown that one of the key modifiers of the effectiveness of aspirin is the timepoint of exposure. By increasing the period of dietary exposure to include *in utero* and perinatal exposure we have dramatically enhanced the ability of aspirin to modify the $Apc^{Min/+}$ phenotype. Within our experimental design, this enhancement is particularly notable given the failure to observe any effect in the $Apc^{Min/+}$ cohort treated post-weaning.

These findings are consistent with the presence of a developmental 'window' for adenoma formation either *in utero* or shortly after birth. In support of this, Shoemaker *et al.,* (1994) showed that perinatal exposure to chemical carcinogens specifically enhances intestinal tumour development in the adult. One possible explanation for this phenomenon is the huge expansion in the number of intestinal crypts which occurs three weeks after birth. This process of crypt fission inevitably expands the number of any mutation-bearing crypts present within the intestine and may therefore clonally expand any crypts bearing mutations at the *Apc* locus.

The presence of a very early 'window' for neoplastic development raises serious questions about our understanding of *Apc*-mediated tumorigenesis. Loss of *Apc* function is considered to be the key initiating event in intestinal neoplasia and is strongly associated with both dysplasia and dysregulation of β-catenin (e.g. Kongkanuntn *et al.,* 1999). However these phenomena are simply not observed in mice aged less than four weeks, indicating either that loss of *Apc* alone is insufficient to lead to the upregulation of β-catenin and development of dysplasia, or that loss of *Apc* (and thereby dysregulation of β-catenin) occurs as a secondary event. In this regard it has previously been shown that a proportion of dysplasias occurring in a mismatch repair deficient background are not characterised by increased beta-catenin levels (Kongkanuntn *et al.,* 1999).

Aspirin clearly acts to suppress these early events either by deleting or suppressing the phenotype of those cells which carry an increased predisposition to adenoma formation. This seems unlikely to be through modulation of *β*-catenin levels, as has been suggested by Mahmood *et al.,* (1998a, 1998b), because, as stated above, elevated levels of *β*-catenin are not observed in young animals. A second possibility is that aspirin may suppress neoplasia through a Cox2 mediated pathway. Cox2 upregulation is observed in 80-85% of human colorectal carcinomas, 50% of colorectal adenomas and within tumours arising in the Apc^{Min} model (Williams *et al.,* 1996). However, it seems unlikely that the primary effect of *in utero* aspirin exposure is mediated through Cox2 suppression, as Cox2 overexpression is associated with the

later stages of tumorigenesis by modulating angiogenesis and levels of apoptosis within tumours (Tsujii and Dubois 1995, Tsujii *et al.,* 1998).

Whatever the mechanism of action of aspirin, these studies demonstrate that initiation of adenoma formation occurs very early in the Apc^{Min} mouse and that aspirin exposure at this time point can efficiently suppresses this process.

Given aspirins reported ability to down regulate β-catenin in tumours, the effect of single high dose aspirin is currently being investigated. In the last chapter it was shown that β-catenin upregulation occurs at very early stages of intestinal neoplasia in the $Apc^{Min/+}$ mice. Therefore in a similar study to the previous chapter, the effect of aspirin upon apoptosis and cell cycle (via BrdU incorporation) in both normal intestine and intestinal lesions is being performed. This could raise the possibility that NSAIDs could work in chemotherapy as well as chemoprevention.

Aspirin also delayed tumourigenesis in $Msh2^{-/-}$ background (albeit weakly), and in the ($Apc^{Min/+}$, $Msh2^{-/-}$) mice. Overall, the median lifespan of mock exposed $Apc^{Min/+}$ mice was 175 days compared to 330 days of aspirin treated mice, an increase of 89% or 155 days. The comparable figures for the ($Apc^{Min/+}$, $Msh2^{-/-}$) cohort are 98 days (mock treated) and 127 days (exposed), an increase of 29% or 29 days. This comparison suggests that the effect of aspirin was no greater in the ($Apc^{Min/+}$, $Msh2^{-/-}$) background. Taken together with the weak effect of aspirin on survival of $Msh2^{-/-}$ mice and the failure to observe an influence of aspirin upon mutation rate, these data argue that aspirin specifically suppresses tumourigenesis in an *Apc* dependent manner. This conclusion contrasts the observation that *in vitro* aspirin can delete cells characterised by MSI (Ruschoff *et al.,* 1998). This contradiction may be explained by the surprising fact that adenomas developing in ($Apc^{Min/+}$, $Msh2^{-/-}$) mice do not show microsatellite instability (Reitmair *et al.,* 1996a, 1996b). This demonstrates that gross microsatellite instability is not required to drive adenoma formation in this background, possibly because of the high selective pressure for adenoma formation consequent upon the Apc^{Min} mutation. Thus, somewhat paradoxically, cells within the ($Apc^{Min/+}$, $Msh2^{-/-}$) mice do not exhibit gross

levels of MSI and therefore are not available as targets for aspirin mediated deletion. The weak suppression of lymphomagenesis on the *Msh2*$^{-/-}$ background could also reflect the fact that only 50% of *Msh2*$^{-/-}$ lymphomas show MSI (see chapter 6). This is in contrast to intestinal tumours from HNPCC patients, where all tumours show MSI. Thus there is still the possibility that NSAIDS could work even more effectively on these tumours with MSI.

In addition to suppressing intestinal neoplasia, aspirin exposure also significantly delays the onset of mammary tumorigenesis in the (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) mice. This is consistent with several recent studies, which have shown that both mutation of *Apc* and over-expression of Cox2 can lead to mammary neoplasia (Liu *et al.,* 2001), and furthermore that selective Cox2 inhibitors can suppress rodent mammary tumourigenesis (Alshafie *et al.,* 2000). At present pancreatic neoplasia is being scored in these *Apc*$^{Min/+}$ and (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) mice to see whether this is also delayed (Clarke *et al.,* 1995). This raises the possibility that NSAIDS could be effective on a range of different tumours with *Apc* mutations and upregulation of Cox2.

In summary, it has been shown that dietary aspirin exposure can suppress tumourigenesis in the murine intestine and the mammary gland. This effect appears to be specifically associated with loss of function of *Apc* and appears to only weakly modify the MMR phenotype. Critically, in the intestine suppression only becomes apparent if exposure covers the period between conception and weaning. Obviously. prophylactic treatment with aspirin of FAP patients during this window would be inappropriate due to the well-known deleterious side effects of aspirin. This therefore identifies a potential window of opportunity for the chemoprevention of intestinal neoplasia. The challenge will now to establish whether NSAIDS (other than aspirin) can be therapeutically effective within this window.

Chapter 11: Summary

This section gives a brief overview of the main conclusions of this thesis.

First, that apoptosis following DNA damage is dependent on a number of genes. I have shown that this gene dependent apoptosis is agent type specific and in some circumstances dose dependent. The levels of the reduction in apoptosis in the knockout mice are again gene dependent. For example, loss of *p53* virtually abrogates the immediate wave of apoptosis, whilst loss of *Msh2* can cause a much smaller reduction. Similarly, the kinetics of the apoptotic response differ with a delayed wave of apoptosis observed in the $p53^{-/-}$ nulls, but not in the other singly mutant mice.

Second, that the loss of a particular gene dependent apoptosic response does not necessarily lead to increased long term survival nor increased mutation accumulation. Again this appears agent type specific. I have shown that loss of *Msh2 in vivo* causes reduced apoptosis, increased survival and mutation following alkylation damage. This agrees with the simple hypothesis that loss of apoptosis causes long term accumulation of mutant cells that can predispose to malignancy. However this is very much the exception to the general trend whereby failure to engage apoptosis does not predict either survival or mutation accumulation. One example of this is that loss of $Mbd4^{-/-}$ causes both a reduction in the apoptotic response to cisplatin and enhanced long term survival, yet it does not increase mutation accumulation. Therefore a central conclusion of this thesis is that failure to engage apoptosis cannot be used as a direct predictor of long term cell fate.

Third, that a reduction in apoptosis correlates with progression of intestinal neoplasia in the $Apc^{Min/+}$mouse. However this relationship is more complicated than first reported by Bedi *et al.,* (1995), with small single crypt (type I) lesions showing increased levels of apoptosis compared to normal epithelium. This therefore supports the widely believed hypothesis (which in fact has little direct

evidence to support it) that loss of the apoptotic response is an important factor in tumour progression.

Fourth, that gene dependency of the apoptotic response is retained within tumours. This has clear ramifications for treatments that are thought to work by engaging apoptosis, given the high frequency of *p53* mutations in colorectal cancer and poor understanding of how precisely chemotherapy works. From the data presented here it seems that we are still faced with a significant challenge in understanding the response of a system (e.g. a tumour) in terms of individual cellular responses (e.g. apoptosis). Thus, although the development of new therapies based on single gene functions appear potentially very logical, the need to understand basic tumour biology still persists.

Finally, the $Apc^{Min/+}$ and the ($Apc^{Min/+}$, $Msh2^{-/-}$) murine models of intestinal malignancy were used to test the efficacy of aspirin for chemoprevention. Aspirin was shown to dramatically suppress intestinal malignancy when adminstered *in utero*. Given aspirin's reported ability to down regulate β-catenin and the findings reported here linking high β-catenin expression with apoptosis, it will be of great interest to determine whether aspirin mediates its effect through gene dependent apoptosis (Mamhoud *et al.,* 1998a).

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13.0 Appendix: Cytotoxic agents and gene dependency

This final section gives a brief overview of the genotoxic drugs that will be used in this thesis.

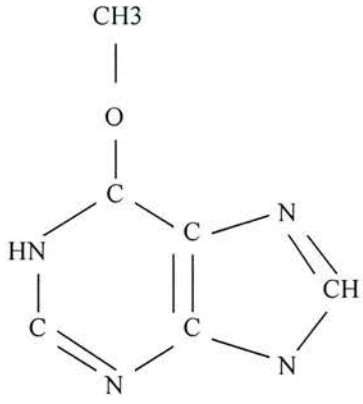
13.1 Methylating Agents : Temozolomide, MNNG, MNU

The cytotoxic lesion produced by all of these drugs is thought to be the 0^6meG. It elicits both *Msh2* and *p53* dependent apoptosis (Toft *et al.* 1999). Both NMNU and temozolomide have been used in chemotherapy. At present temozolomide is being used to treat malignant glioma in the US (Friedman *et al.* 2000). However for all three of these drugs the most predominant lesions are N7 methyl guanine and N3 methyl adenine. These are thought to repaired very quickly by the base excision repair genes : N7-methylguanine and N3 methyl adenine DNA glycosylases (Newlands *et al.* 1997). The evidence that 06 methylguanine is the cytotoxic lesion comes form studies using NMNU as a carcinogen. Mice treated with NMNU can be protected from tumourigenesis by over expression of O6-alkylguanine-DNA alkyltransferase (MGMT). MGMT is a suicide transferase which removes the 06 meG and to a lesser extent O4 meT. Thus increases in the level of this enzyme protects against tumourigenesis (Dumenco *et al.* 1993, Allay *et al.* 1999). Likewise reduction of levels of MGMT causes increased sensitivity and apoptosis to these agents (Tentori *et al.* 1997, Bignami *et al.* 2000).

Figure 13.1. Percentage lesion profile of the methylating agents: Adapted from Pieper 1995, Eisenbrand *et al.* (1994), Goldmacher *et al.* (1986) and Denny *et al.* (1994). In case where numbers of lesions do not add up to 100% (e.g. temozlomide) this is due to the study only quantifying a certain lesion type (Denny *et al.* 1994).

Lesion	NMNU	MNNG	Temozolomide
Adenine:			
N1	0.9	0.2	
N3	8.4	3.7	
N7	2.0	3.4	
Guanine			
N3	0.6	9.7	
O6	6-10	9.2	5
N7	66.4	74.8	70
Cytosine			
02	0		
N3	0.5		
Thymine			
O2	0.1	0.2	
N3	0		
O4	0.7	07	
Phosphodiester	12.1	nd	

Figure 13.2. Structure of O6 methyl Guanine (O6meG). Although the O6 position of guanine is a weak nucleophile and reacts with the methylating agents much less than the N7 lesion, it's the most important adduct for both mutagenesis and carcinogenesis.



13.2 Cisplatin damage

Cisplatin (cis-diamminedichlorolplatinum II) is used very successsfully in the treatment of testicular cancer. It is also used in the treatment of ovarian and bladders cancers (Brown *et al.* 1997).

Figure 13.3: Structure of cisplatin and transplatin (adapted from Leng and Brebec 1994)

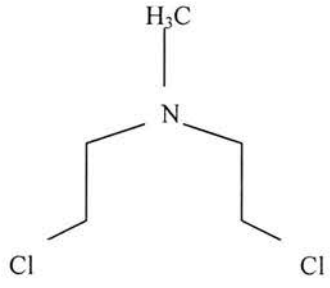
Cisplatin Transplatin

Insights into the cytotoxic adducts have come from comparison of the adducts formed between cisplatin and transplatin. Unlike cisplatin, transplatin (an isomer of cisplatin) does not appear to be cytotoxic (Zlatnova *et al.* 1998). The major difference is that predominant lesion (approx 65%) cisplatin produces is an cis GG intrastrand crosslink. This induces a large bend in the DNA, blocking transcription. It is thought that the major pathway for cisplatin removal is through nucleotide excision repair with mismatch repair playing a smaller role (Zlatanova *et al.* 1998). Cisplatin has also been shown to induce apoptosis (probably through the G-G lesion) which is p53 dependent (Toft *et al.* 1999). Recently Branch *et al.* (2000) showed in ovarian cell lines that resistance to cisplatin predominantly related to p53 status with mismatch repair playing a much smaller role.

13.3 Nitrogen Mustard

Nitrogen mustards are among the oldest chemotherapeutics still being used. They can form large bulky adducts and crosslinks that block DNA replication (Hemminiki 1994). In fact the nitrogen mustard melphalan produces a very similar set of lesion to cisplatin. The nitrogen mustard used here is nitrogen mustard (Mechlorethamine Hydrochloride). Fan *et al.* (1997) showed that both p21 null MEFS and HCT116 colorectal cancer cells showed preferential sensitivity to Nitrogen Mustard. Nitrogen Mustard has been shown to induce apoptosis and kill small intestinal stem cells (Ijiri and Potten 1984, 1987). In this thesis the p53 and the MMR dependency of this apoptosis will be investigated.

Figure 13.4. Structure of Nitrogen Mustard



13.4 Additional Methods

The methods described in the following section were performed by Lucy Curtis and Neil Toft. Data from these methods are included in chapter 6.

Immunophenotyping of thymic lymphomas

Fresh tumour tissue was dissagregated and frozen in 1ml freezing medium. Prior to analysis samples were defrosted and lymphocytes isolated using Lympholyte M (Cedarlane Laboratories) as per protocol. Lymphocytes were resuspended in Flow Buffer [1% Bovine Serum Albumin, 1% Sodium Azide] and incubated with primary antibody on ice for 1 hour, washed and incubated with 25µl of the appropriate FITC secondary antibody for a further hour. Cells were resuspended in 200µl of Flow Buffer before analysis on the EPICS XL Flow Cytometer using XL2 Software (Coulter).

Analysis of wild-type p53 allele in MSH2$^{-/-}$ p53$^{+/-}$ mice

DNA was extracted from small pieces (3mm x 3mm) of tumour using phenol / chloroform extraction and resuspended in 500µl TE and *p53* status determined by PCR Total RNA was extracted using 1ml Trizol (GibcoBrl). 1µl of RNA was reverse transcribed into cDNA using oligo$(dT)_{15}$ primers. PCR primers 33A and 21B (Ozbun *et al.* 1993) were used to amplify a 686 bp fragment of *p53* cDNA which included exons 5-9. For each tumour cDNA three separate PCR reactions were performed. Each PCR product was cloned and sequenced from both directions using a Fluorescent Automated DNA Sequencer (Licor).

DNA flow cytometry

DNA content of tumours was analysed according to Vindelov *et al.* 1983. DNA content was analysed on a EPICS XL Flow Cytometer using Multicycle Software (Coulter).

Comparative Genomic Hybridisation (CGH)

CGH was carried out on a series of 42 lymphomas according to the method of Kallioniemi *et al.,* (1992), slightly modified. Briefly, DNA was labelled by incorporation of digoxygenin-11-dUTP (normal DNA) or biotin-16-dUTP (test DNA) by nick translation. Metaphase spreads were prepared from karyotypically normal E14 mouse embryonic stem cells. Slides were pretreated with 10μg/ml RNase A at 37°C for one hour and 100ng/ml proteinase K solution at 37°C for 2.5 minutes, denatured in 70% formamide/2XSSC solution at 70°C for 3 minutes and dehydrated through a ethanol series. 500ng of each labelled probe and 20μg mouse Cot-1 DNA were denatured at 70°C for 5 minutes and allowed to reanneal at 37°C for 1 hour before hybridisation onto slides for 2 days. Detection was carried out with FITC-avidin and antidigoxygenin-rhodamine and chromosomes counterstained with DAPI as described (Kallioniemi *et al.,* 1992). Hybridisations were analysed using Quantitative Image Processing System (QUIPS) software (Vysis Ltd, Richmond, UK). For each tumour, four to eight metaphase spreads were karyotyped manually and green/red ratio cut-off points of 1.2 and 0.8 were chosen for scoring of chromosome copy number changes.

Microsatellite analysis of mouse tumours

Four microsatellite loci were chosen for analysis: D1Mit4, D7Mit17, D10Mit2 and D14Mit15. Primers sequences and reaction conditions were obtained from the Whitehead Institute for Biomedical Research/Massachusetts Institute of Technology Centre for Genome Research website at http://www.genome.wi.mit.edu. MSI was assessed by comparison of normal and tumour PCR products on a 6% denaturing polyacrylamide gel with silver staining, as described elsewhere (Bubb *et al.* 1996).

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Advances in Brief

Suppression of Intestinal and Mammary Neoplasia by Lifetime Administration of Aspirin in *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$, *Msh2*$^{-/-}$ Mice[1]

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Abstract

Numerous studies have indicated that exposure to nonsteroidal anti-inflammatory drugs is associated with a lowered risk of colorectal cancer. However, analyses of the effect of aspirin upon tumorigenesis in *Apc*$^{Min/+}$ mice have yielded contrasting results. We show that adult dietary exposure to aspirin does not suppress intestinal tumorigenesis in *Apc*$^{Min/+}$ mice, but that continual exposure from the point of conception does. To test whether this regime could suppress the phenotype of murine models of hereditary nonpolyposis colorectal carcinoma, *Msh2*-deficient mice were exposed to aspirin. This did not modify the mutator phenotype of *Msh2*$^{-/-}$ mice, but weakly extended survival. Finally, we analyzed (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) mice and found that lifetime aspirin exposure significantly delayed the onset of both intestinal and mammary neoplasia. Thus embryonic and perinatal exposure to aspirin suppresses neoplasia specifically associated with the loss of *Apc* function, opening a potential window of opportunity for nonsteroidal anti-inflammatory drug intervention.

Introduction

Substantial epidemiological evidence shows that treatment with NSAIDS[3] reduces the risk of developing colorectal cancer. The most commonly used NSAID has been aspirin, which has been reported to reduce the risk of colon cancer by up to 40%. Clinical studies using the NSAID sulindac have also reported reduced polyp counts in FAP patients (1, 2). The precise mechanism of NSAID action remains unclear, although the suppression of Cox2 is thought to be of pivotal importance. Significantly, Cox2 is overexpressed in ~85% of human colorectal adenocarcinomas and adenomas from the *Apc*^{Min} mouse (3). The definitive study highlighting the relevance of Cox2 to tumorigenesis showed that a Cox2-deficient background markedly suppressed intestinal neoplasia in mice carrying the *Apc*^{8716} allele (4). The biological activities of aspirin and sulindac are not restricted to suppression of the Cox2 pathway. Aspirin, sulindac, and even the selective Cox2-inhibitor celecoxib (5) have been shown to induce apoptosis in a Cox2-independent fashion. Both aspirin and sulindac down-regulate β-catenin- and β-catenin/TCF4-mediated transcription (6, 7). NSAIDS also act on the nuclear factor κB signaling pathway (8), and recent data suggest this may be important for their antitumor activities (8). They have also been shown to specifically reduce the survival of genetically unstable (MSI+) MMR-deficient colorectal cancer cell lines (9), raising the possibility that aspirin may also suppress malignancy in hereditary nonpolyposis colorectal carcinoma families characterized by mutations in the *MMR* genes. Mice constitutively heterozygous for the *Apc*^{Min} allele have been used to determine the ability of the NSAIDs to suppress intestinal malignancy. However with respect to aspirin, these studies have produced contrasting results. Virtually every study [*e.g.*, Beazer-Barclay *et al.*, (10) has shown that sulindac causes a reduction in the number of the spontaneous malignancies, apart from Oshima *et al.* (4) who use relatively low levels of sulindac]. However, of the studies that have investigated spontaneous intestinal malignancy after aspirin treatment, only two have shown suppression of malignancy in the Apc^{Min} mouse. Two other studies failed to show suppression in either the *Apc*^{Min} mouse or the *Apc*^{1638N} mouse (6, 11–13). The basis for these discrepancies may lie within differences in the aspirin regime used. Shoemaker *et al.* (14) and Reitmair *et al.* (15) have argued that the majority of adenomas are fixed either *in utero* or perinatally just after birth.

Here we directly test the effect of increasing the period of aspirin exposure to include the entire period from the point of conception onwards. Furthermore, we have investigated whether this regime can modify the development of MMR-associated neoplasia by investigating the course of neoplasia in cohorts of *Msh2*$^{-/-}$ and (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) mice. We find that prolonged aspirin exposure dramatically enhances the suppression of *Apc*-associated neoplasia both within the intestine and the mammary gland but only weakly influences the phenotype of MMR deficiency.

Materials and Methods

Administration of Dietary Aspirin at Weaning. C57BL/6 mice wild-type and heterozygous for the *Apc*^{Min} mutation were placed on diets containing either 0, 200, or 400 mg/kg of aspirin (Harlan/Tekad). These levels of exposure are comparable with the highest doses used in previous studies (6, 11–13). Mice were monitored everyday for signs of disease, generally manifesting itself as anemia, loss of weight, and a hunched appearance.

Two experiments were performed: one where mice were killed at 150 days of age and one where mice were killed where they showed signs of disease.

Permanent Administration of Aspirin. Matings segregating for progeny which were *Apc*$^{Min/+}$, *Msh2*$^{-/-}$ or (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) were placed on aspirin diets containing either 0 or 400 mg/kg of aspirin before conception and throughout pregnancy and lactation. Progeny were weaned onto appropriate diets. Mice were killed when they showed signs of disease.

Histological Analysis. Tissues were removed, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 10 um, and stained with H&E before microscopic analysis. Scoring of intestinal lesion was achieved by removing the entire intestine at necropsy, flushing with PBS, and mounting *en face*. These preparations were then fixed in methacarn (4:2:1, methanol: chloroform:glacial acetic acid). Lesions were then scored macroscopically. Intestine was then wound into a "swiss" roll, which was subsequently embedded in paraffin and then sectioned as above.

Mutation Frequency at the *Dlb-1b* Locus. The *Dlb-1* assay was performed as described previously (16). For this assay, experimental cohorts were derived by backcrossing the *Msh2* mutants to two different C57Bl/6 strains, one of which was homozygous for the *Dlb-1 a* allele and one of which was homozygous for the *Dlb-1 b* allele. Mice were subsequently intercrossed from

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[3] The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; FAP, familial adenomatous polyposis; Cox2, cyclooxygenase 2; Dlb-1b, dolichos biflorus.

these two lines to generate mice heterozygous at the *Dlb-1* locus and segregating for all possible *Msh2* genotypes.

Results and Discussion

Aspirin Does Not Suppress Intestinal Neoplasia when Administered at Weaning. Wild-type and Apc^{Min} mice were placed on aspirin-containing diets (either 200 mg/kg or 400 mg/kg) at weaning. (Fig. 1). Mice exposed to the aspirin diet gained weight normally when compared with mice on the control diet, and no ulceration or intestinal pathology such as perforation of the intestine was observed in any of the wild-type mice treated with aspirin. Two independent analyses were performed.

First, cohorts of mice either wild-type or heterozygous for the Apc^{Min} allele were killed at 150 days of age, and the adenoma burden was assessed on whole mount preparations of the entire small intestine. No adenomas were seen in wild-type mice. Adenoma burden was as follows: for mock treated Apc^{Min} mice (n = 5), 9.8 (±/6.3); for mice treated with 200 mg/kg (n = 7), 9.7 (±5.88); and for mice treated with 400 mg/kg of aspirin (n = 5), 8.2 (±6.05). There was no difference in tumor burden at either 200 mg/kg (P = 1.00) or at 400 mg/kg (P = 0.75; Mann Whitney) compared with mock-treated mice, indicating that aspirin exposure was not modifying the Apc^{Min} phenotype.

Second, cohorts of mice were permitted to age until they developed obvious symptoms of intestinal neoplasia, usually bleeding from the anus or anemia scored through whitening of the feet. Fig. 1*A* shows a Kaplan-Meier plot reflecting survival over a 400-day period. Exposure to aspirin at either 200 mg/kg or 400 mg/kg did not alter the survival profile, again indicating that exposure to aspirin did not modify the Apc^{Min} phenotype.

Aspirin Suppresses Intestinal Neoplasia when Administered *in Utero*. Because Shoemaker *et al.* (14) and Reitmair *et al.* (15) have argued that the majority of adenomas are fixed before 6 days of age, we investigated the effect of aspirin exposure throughout embryogenesis and weaning. Cohorts of wild-type and Apc^{Min} heterozygotes were derived from parents placed on aspirin-containing diets before conception. Dietary exposure to aspirin was maintained throughout and beyond weaning. We first wished to establish whether *in utero* exposure resulted in increased embryonic lethality of Apc^{Min} heterozygotes, as has been reported for the Cox1- and Cox2-inhibitor piroxicam (17). Analysis of offspring showed this not to be the case, because there was no reduction in the number of Apc^{Min} heterozygotes in progeny from aspirin-exposed parents (P = 0.2; χ^2 test).

We next determined the survival profiles of each cohort (Fig. 1*A*), which showed a significant increase in survival in Apc^{Min} heterozygotes exposed to aspirin from conception onwards (P = 0.0004; log-rank test). This effect was sufficient to completely prevent the development of symptoms associated with intestinal neoplasia in 5 of 16 Apc^{Min} at 500 days of age. Analysis of tumor burden and distribution in those mice that developed intestinal tumors showed no obvious differences in either small (Fig. 1*B*) or large intestine (data not shown).

A number of different possibilities may underlie the discrepancies between the published studies examining the effect of aspirin on Apc^{Min}-associated tumorigenesis. First, that it is a consequence of differences in the level of aspirin exposure—a possibility which seems unlikely given the similar levels used in each experiment. Second, that it is attributable to differences in the genetic background of the mice used, either at known modifiers of the Apc^{Min} phenotype, such as *Mom-1*, or at as-yet unidentified modifiers (18). In this respect, it is notable that our study differs from previous studies by using mice homozygous for the relatively resistant C57/Bl6 *Mom-1* allele. Third, that it is a consequence of different husbandry regimes; for example, mice housed in a sterile facility may respond very differently to those in a nonsterile facility.

Comparison of the data presented here with published experiments does not permit us to distinguish between these possibilities, but it does serve to underline the relatively fragile nature of aspirin-mediated suppression within this model. We show here that one of the key modifiers of the effectiveness of aspirin is the time point of exposure. By increasing the period of dietary exposure to include *in utero* and perinatal exposure, we have dramatically enhanced the ability of aspirin to modify the Apc^{Min} phenotype. Within our experimental design, this enhancement is particularly notable given the failure to observe any effect in the Apc^{Min} cohort treated postweaning.

These findings are consistent with the presence of a developmental "window" for adenoma formation either *in utero* or shortly after birth. In support of this, Shoemaker *et al.* (14) showed that perinatal exposure to chemical carcinogens specifically enhances intestinal tumor development in the adult. One possible explanation for this phenomenon is the huge expansion in the number of intestinal crypts which occurs 3 weeks after birth. This process of crypt fission inevitably

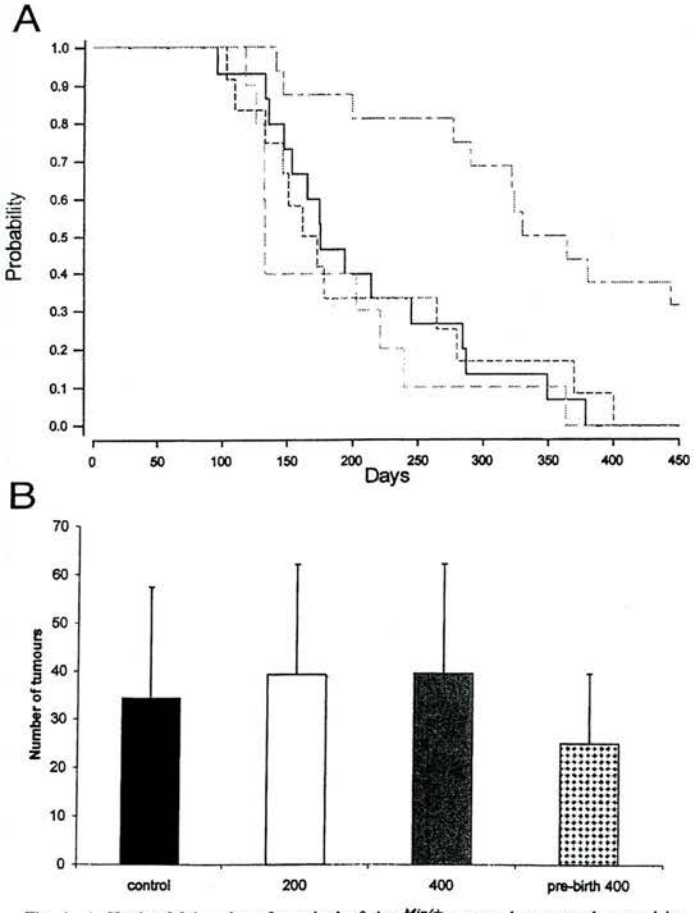

Fig. 1. *A*, Kaplan-Meier plot of survival of $Apc^{Min/+}$ exposed to control or aspirin-containing diet. *Black line*, mock treated mice (n = 15); *red line*, 200 mg/kg diet (n = 12); *blue line*, 400 mg/kg of diet (n = 10). There was no significant difference in the survival curves of either the mice treated with 200 mg/kg (log-rank test; P = 0.96) and 400 mg/kg (log-rank test; P = 0.30). *Green line*, $Apc^{Min/+}$ mice exposed to aspirin-containing diet from point of conception. These mice (n = 16) showed a significant increase in survival (log-rank; P = 0.0004) compared with mock-treated and aspirin-treated mice at weaning. *B*, tumor burden of mice at point of sacrifice as determined by onset of overt symptoms of intestinal tumorigenesis. ■, mock-treated mice; □, mice treated with 200v mg/kg; ▨, mice treated with 400 mg/kg of aspirin at weaning; ▦, mice treated with 400 mg/kg of aspirin from point of conception. There was no significant difference in the number of tumors at sacrifice between mock-exposed mice (n = 15) and mice exposed to 400 mg/kg from conception (Mann-Whitney; P = 0.613; n = 9). Tumor burden of mice >500 days of age were excluded from this analysis, because these mice were killed when symptomatic of lymphoma and not intestinal neoplasia.

expands the number of any mutation-bearing crypts present within the intestine and may therefore clonally expand any crypts bearing mutations at the *Apc* locus.

The presence of a very early window for neoplastic development raises serious questions about our understanding of *Apc*-mediated tumorigenesis. Loss of Apc function is considered to be the key initiating event in intestinal neoplasia and is strongly associated with both dysplasia and dysregulation of β-catenin [*e.g.,* Kongkanuntn *et al.* (19)]. However these phenomena simply are not observed in mice aged <4 weeks, indicating either that loss of *Apc* alone is insufficient to lead to the up-regulation of β-catenin and development of dysplasia, or that loss of *Apc* (and thereby dysregulation of β-catenin) occurs as a secondary event. In this regard we have shown previously that a proportion of dysplasias occurring in a mismatch repair-deficient background are not characterized by increased β-catenin levels (19).

Aspirin clearly acts to suppress these early events either by deleting or suppressing the phenotype of those cells which carry an increased predisposition to adenoma formation. This seems unlikely to be through modulation of β-catenin levels, as has been suggested by Mahmood *et al.* (6), because, as stated above, elevated levels of β-catenin are not observed in young animals. A second possibility is that aspirin may suppress neoplasia through a Cox2-mediated pathway. Cox2 up-regulation is observed in 80–85% of human colorectal carcinomas, in 50% of colorectal adenomas, and within tumors arising in the Apc^{Min} model (3). However, it seems unlikely that the primary effect of *in utero* aspirin exposure is mediated through Cox2 suppression, ~~inasmuch as~~ Cox2 overexpression is associated with the later stages of tumorigenesis by modulating angiogenesis and levels of apoptosis within tumors (3).

Whatever the mechanism of action of aspirin, these studies demonstrate that the initiation of adenoma formation occurs very early in the Apc^{Min} mouse, and that aspirin exposure at this time point can efficiently suppresses this process. Understanding the factors determining the predisposition to adenoma formation during embryonic development and perinatally might lead to greater insights into the molecular mechanisms of cancer in humans.

Aspirin Weakly Suppresses Neoplasia though not Mutation in $Msh2^{-/-}$ Mice. Having established an effective protocol for aspirin exposure in the murine model of FAP, we determined whether this approach could modulate the phenotype of the murine model of hereditary nonpolyposis colorectal ~~carcinoma.~~ cancer. A significant subset of human intestinal tumors are characterized by mutations in the MMR pathway, and all murine models of MMR deficiency show increased predisposition to neoplasia.

Cohorts of $Msh2^{-/-}$ mice were either exposed to aspirin or were fed control diet from conception, and Kaplan-Meier survival curves were generated (Fig. 2*A*). All 18 animals on the control diet were killed after the development of lymphoma, although 2 animals also had coexistent intestinal malignancy. All $Msh2^{-/-}$ mice exposed to aspirin also developed lymphoma; however, there was a small increase in survival compared with controls ($P = 0.05$; log-rank test). This slight shift in survival may reflect weak suppression of either intestinal malignancy or lymphomagenesis. This latter possibility is consistent with the one report that showed that aspirin exposure can result in the deletion of MSI-unstable cells in culture (9).

These results prompted us to determine directly whether aspirin exposure can suppress *in vivo* mutation in a mismatch repair-deficient background. Mutation frequency was scored at the *Dlb-1b* locus in *Msh2* mutant mice continually exposed to either control or aspirin-containing diets. Using this assay, we have shown previously that *Msh2*-deficient mice have a mutator phenotype at the *Dlb-1b* locus (16, 20). *Msh2*-deficient mice were analyzed at 4 months of age after exposure to either control or aspirin-containing diet. No difference in mutation frequency was observed at this time point, demonstrating that this regime of aspirin exposure does not modify the mutator phenotype of *Msh2*-null epithelium (Fig. 2*B*).

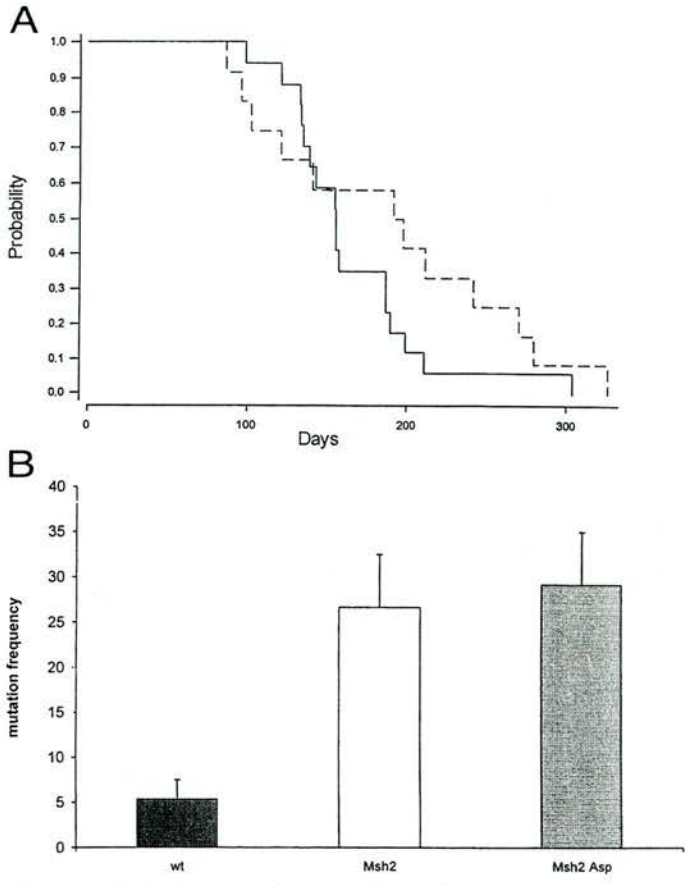

Fig. 2. *A,* Kaplan-Meier plot of survival of $Msh2^{-/-}$ mice exposed to control or aspirin-containing diet from point of conception. ———, mock-treated $Msh2^{-/-}$ mice ($n = 18$); ----, 400 mg/kg-treated $Msh2^{-/-}$ mice ($n = 20$). $Msh2^{-/-}$ mice treated with aspirin showed a weak increase in survival (log-rank test; $P = 0.05$). *B, in vivo* mutation frequency/10,000 villi at the *Dlb-1b* locus after aspirin treatment. Mutation frequencies were determined from intestinal wholemounts at 4 months of age. *Columns,* mean mutation frequency at the *Dlb-1b* locus. At least three mice were used per each column. *Bars,* SD. ■, mock-treated wild-type mice; □, mock-treated $Msh2^{-/-}$ mice; ▩, $Msh2^{-/-}$ mice exposed to 400 mg/kg of dietary aspirin from point of conception. There was not a significant reduction in mutation frequency after aspirin treatment ($P = 0.77$; Mann-Whitney).

Aspirin Suppresses Intestinal and Mammary Neoplasia in $Apc^{Min/+}$ $Msh2^{-/-}$ Mice. In human colorectal cancer MMR-deficient tumors differ in a number of respects to MMR-proficient tumors. For example, MMR-deficient tumors express lower levels of Cox2 as well as exhibiting very different patterns of mutation and genomic instability. These differences invoke different mechanisms for MMR-driven neoplasia, raising the possibility that aspirin exposure may have an additional effect in a MMR-deficient background, for example through the deletion of cells showing MSI.

The development of lymphoma in $Msh2^{-/-}$ mice largely precludes an analysis of the effect of aspirin upon *Msh2*-dependent intestinal neoplasia (22). However, *Msh2* deficiency has been shown to greatly accelerate intestinal neoplasia in the Apc^{Min} mouse (15), so permitting an analysis of the effect of aspirin in this context. In addition, mammary akanthoma occur in $Apc^{Min/+}$ mice (23), and this is greatly accelerated in ($Apc^{Min/+}$, $Msh2^{-/-}$) mice, permitting a study of the effect of aspirin on the development of this lesion (see below). Cohorts of ($Apc^{Min/+}$, $Msh2^{-/-}$) mice were generated and exposed to either control diet or aspirin-containing diet from conception onwards, and Kaplan-Meier survival curves were generated (Fig. 3*A*). Survival was markedly enhanced after aspirin expo-

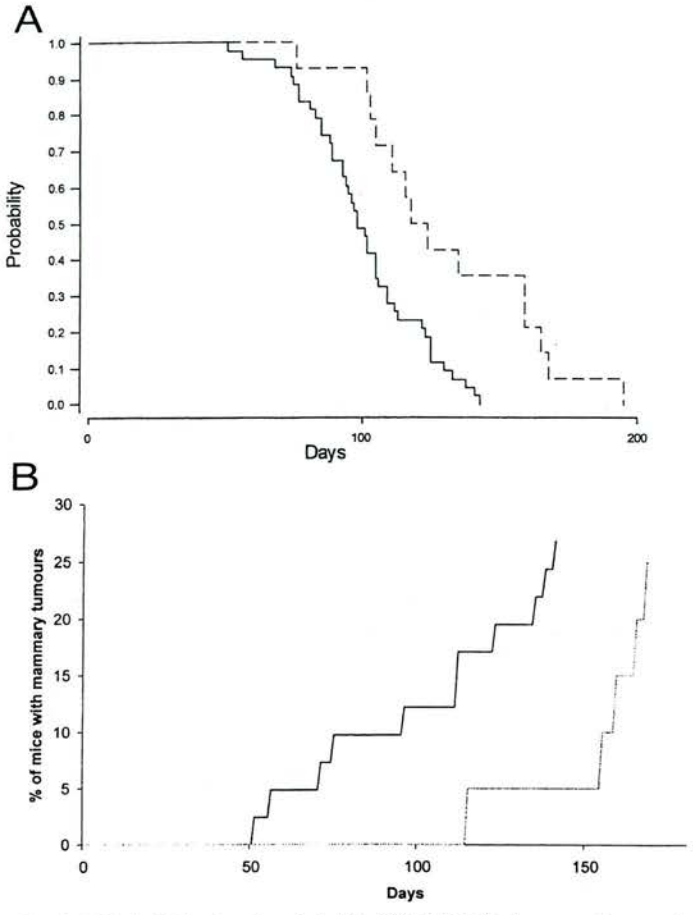

Fig. 3. *A*, Kaplan-Meier plot of survival of $Apc^{Min/+}$ $Msh2^{-/-}$ mice exposed to control or aspirin-containing diet from point of conception. ———, mock-treated mice (n = 43); ----, mice treated with 400 mg/kg from conception (n = 17). Aspirin treatment significantly increased survival (log-rank test; P = 0.0001). *B*, graph showing delayed incidence of mammary tumorigenesis in the Min Msh2 mice. ———, mock-treated mice; , mice treated with 400 mg/kg of aspirin from conception.

sure (log-rank test; P = 0.0001), although it was still reduced by comparison to untreated Apc^{Min} heterozygotes. Aspirin exposure did not alter either tumor distribution or burden scored at the point of death (data not shown).

In the cohorts maintained on the control diet, a single mammary akanthoma was observed in a total of 15 $Apc^{Min/+}$ mice, whereas none were detected in 18 $Msh2^{-/-}$ mice. Development of this tumor type was enhanced in $Apc^{Min/+}$ $Msh2^{-/-}$ mice (11 tumors in 41 mice). In the cohorts exposed to aspirin, no mammary akanthomas were observed in 10 $Apc^{Min/+}$ mice, whereas a single akanthoma was observed in 21 $Msh2^{-/-}$ mice. In the ($Apc^{Min/+}$, $Msh2^{-/-}$) cohort, five mammary akanthomas were observed in 20 mice. All of these mice also had intestinal neoplasia. Although this represents a similar prevalence to that observed in the untreated ($Apc^{Min/+}$, $Msh2^{-/-}$) cohort, the onset of akanthoma was significantly retarded in the aspirin-treated mice (Fig. 3*B*). These results therefore show aspirin exposure significantly delays the onset of mammary tumorigenesis in the ($Apc^{Min/+}$, $Msh2^{-/-}$) background, consistent with several recent studies which have shown that both mutation of *Apc* and overexpression of Cox2 can lead to mammary neoplasia (24), and, furthermore, that selective Cox2 inhibitors can suppress rodent mammary tumorigenesis (25).

Overall, the median life span of mock-exposed Apc^{Min} mice was 175 days compared with 330 days for aspirin-treated mice, an increase of 89% or 155 days. The comparable figures for the ($Apc^{Min/+}$, $Msh2^{-/-}$) cohort are 98 days (mock-treated) and 127 days (exposed), an increase of 29% or 29 days. This comparison suggests that the effect of aspirin was no greater in the ($Apc^{Min/+}$, $Msh2^{-/-}$)background. Taken together with the weak effect of aspirin on survival of $Msh2^{-/-}$ mice and the failure to observe an influence of aspirin upon mutation rate, these data argue that aspirin specifically suppresses tumorigenesis in an *Apc*-dependent manner. This conclusion contrasts the observation that *in vitro* aspirin can delete cells characterized by MSI (9). This contradiction may be explained by the surprising fact that adenomas developing in ($Apc^{Min/+}Msh2^{-/-}$) mice do not show microsatellite instability (15), which was independently confirmed here (data not shown). This demonstrates that gross microsatellite instability is not required to drive adenoma formation in this background, possibly because of the high selective pressure for adenoma formation consequent upon the Apc^{Min} mutation. Thus, somewhat paradoxically, cells within the ($Apc^{Min/+}Msh2^{-/-}$) mice do not exhibit gross levels of MSI and therefore are not available as targets for aspirin-mediated deletion.

In summary, we have shown that dietary aspirin exposure can suppress tumorigenesis in the murine intestine and the mammary gland. This effect seems to be specifically associated with a loss of function of Apc and seems to only weakly modify the MMR phenotype. Critically, in the intestine, suppression only becomes apparent if exposure covers the period between conception and weaning. Obviously prophylactic treatment with aspirin of FAP patients during this window would be inappropriate because of the well-known deleterious side effects of aspirin. This, therefore, identifies a potential window of opportunity for the chemoprevention of intestinal neoplasia. The challenge will now be to establish whether NSAIDS (other than aspirin) can be therapeutically effective within this window.

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Msh-2 suppresses *in vivo* mutation in a gene dose and lesion dependent manner

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Mice deficient for the mismatch repair (MMR) gene *Msh2* show accelerated tumourigenesis and a reduced apoptotic response to DNA damage of methylation type. Here we examine the effect of mutation for *Msh2* on *in vivo* mutation frequencies in the intestine as determined by loss of function at the *Dolichos biflorus* (*Dlb-1*) locus. Spontaneous mutation frequencies were scored in cohorts of ageing mice either wild type or mutant for *Msh2*. In mice less than 1 year old, mutation frequencies were only elevated in *Msh2* null mice. However, beyond this age heterozygous *Msh2* mice showed significantly higher mutation frequencies than controls. These findings implicate a gene dose dependent requirement for *Msh2* in mutation suppression and prompted an analysis of young *Msh2* mutants following exposure to DNA damage. Following exposure to N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), *Msh2* deficient mice show a reduced apoptotic response and an increase in mutation frequency. Heterozygotes did not differ from controls. Following exposure to cisplatin, no significant elevation was seen in mutation frequencies, even within homozygotes. This is particularly surprising given the association between cisplatin resistance and MMR deficiency. These findings therefore demonstrate a complex reliance upon functional *Msh2* in mutation surveillance. We have identified three separate scenarios. First, where retention of both *Msh2* alleles over an extended period of time appears critical to the suppression of spontaneous mutation; second, 3 weeks following exposure to MNNG, where only complete loss of *Msh2* results in elevated mutation; and finally following cisplatin exposure, where induced levels of mutation are independent of *Msh2* status. *Oncogene* (2001) **20,** 3580–3584.

Keywords: mismatch repair; apoptosis; mutation; heterozygosity; cisplatin; alkylation

Structural distortions produced by nucleotides which are either unpaired or paired with non-complementary nucleotides are recognized by proteins encoded by the mismatch repair genes. Several members of this family of genes have been characterized within *Saccharomyces cerevisiae*, which led to the identification of mammalian homologues. Six human mismatch repair genes have been cloned, MSH2, MLH1, PMS1, PMS2, MSH3 and MSH6 (Kinzler and Vogelstein, 1996; Wheeler *et al.*, 2000). Constitutive inactivation of these genes has been associated with the development of cancer, the best characterized relationship being between the inherited cancer-susceptibility syndrome of Hereditary Nonpolyposis Colorectal Cancer (HNPCC) and germline mutations in MSH2, MLH1 and PMS2 (Nicolades *et al.*, 1994; Kinzler and Vogelstein, 1996). Mice have been produced bearing targeted inactivations of the *Mlh1*, *Msh2*, and *Pms2* genes. Homozygous mice of these mutant strains are viable but prone to the development of different types of neoplasia. All of these mutant strains develop lymphoma, however they differ in their susceptibility to intestinal neoplasia, which only develops in *Mlh1* and *Msh2* deficient mice (De Wind *et al.*, 1995; Reitmair *et al.*, 1996; Prolla *et al.*, 1998). As such, *Msh2* and *Mlh1* homozygotes can be considered reasonably good models of HNPCC. By contrast, mice heterozygous for *Msh2* and *Mlh1* do not show reduced survival compared to wild type controls, nor are they predisposed increased intestinal neoplasia. They are, however characterized by increased tumorigenesis. Surprisingly when these tumours were examined for Loss of Heterozygosity (LOH) only one out of 71 had lost the remaining copy of *Msh2* and this was the only tumour to exhibit microsatellite instability (MI), indicating that the majority of tumours retained heterozygosity for *Msh2* (De Wind *et al.*, 1998). These results are therefore consistent with a heterozygous effect of Msh2.

There are currently two possible biological mechanisms underlying the relationship between MMR deficiency and neoplasia. The first of these arises out of the MMR proteins defining role in mediating DNA repair. MSH2 recognizes mismatched nucleotides as a heterodimer with MSH6 and insertion/deletion loops with MSH3 (Buermeyer *et al.*, 1999). In what is assumed to be a direct consequence of failure of these processes, *Msh2*−/− cells exhibit a mutator phenotype and microsatellite instability (De Wind *et al.*, 1995; Reitmair *et al.*, 1997). These data support the concept

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that failed recognition and repair of mismatch lesions leads directly to an increase in mutation frequency and thereby to malignancy. A second possible mechanism derives from recent studies which have shown that loss of the mismatch repair gene *Msh2* impairs the apoptotic response to alkylating agents in a variety of systems (Toft *et al*., 1999; Hickman and Samson, 1999). Consistent with this, cells deficient in MMR have been shown to be resistant to a number of clinically important drugs, such as cisplatin, temozolomide and doxorubicin (Fink *et al*., 1998). This raises the possibility that increased mutation frequencies may partly arise from failed clearance of DNA damage bearing cells. Currently, there is little direct evidence in support of this hypothesis, although it is clear that *Msh2* deficiency does lead to an elevation in mutation frequencies following exposure to DNA damage (Andrew *et al*., 1998; Toft *et al*., 1999). Toft *et al*. (1999) have demonstrated that (in a model system) a component of this increase is attributable to increased cell survival. Thus, although Msh2 clearly plays a role in mediating cell death, the precise relationship between the ability to engage apoptosis and mutation surveillance remains relatively poorly defined.

To investigate the *in vivo* consequences of heterozygosity at the *Msh2* locus and to further explore the relationship between apoptosis and mutation surveillance, we have examined the gene dependency of these two endpoints in the small intestine of mice wild type, heterozygous and nullizygous for *Msh2*. These studies were performed both at spontaneous levels of damage and following exposure to the DNA damaging agents MNNG and cisplatin.

We first scored mutation frequency at the *Dlb-1* locus at spontaneous levels of DNA damage over a 13 month time course (Figure 1). The *Dlb-1* assay was performed as previously described (Winton *et al*., 1988). For this assay, experimental cohorts were derived by backcrossing the *Msh2* mutants to two different C57Bl/6 strains, one of which was homozygous for the *Dlb-1a* allele and one of which was homozygous for the *Dlb-1b* allele. Mice were subsequently intercrossed from these two lines to generate mice heterozygous at the *Dlb-1* locus and segregating for all possible *Msh2* genotypes. Loss of the *Dlb-1b* allele was then scored per 10 000 villi.

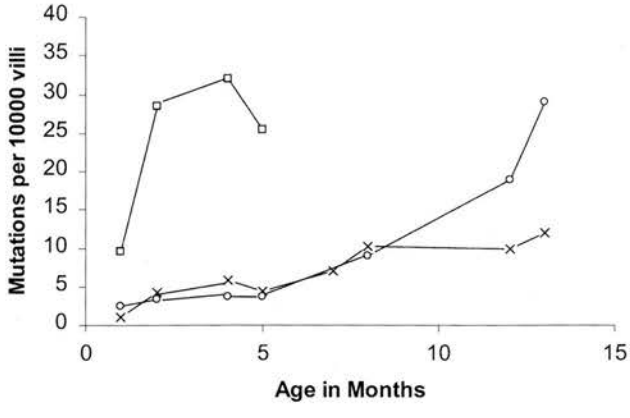

Figure 1 Spontaneous Dlb-1 mutation frequencies scored per 10 000 villi in mice wild type (crosses), heterozygous (circles) and homozygous null (square points) for *Msh2*. Each point represents at least three mice. At ages over 1 year, heterozygous mice ($n=12$, mean = 24) have a significantly higher mutation frequency than wild type ($n=8$, mean = 11) mice. $P=0.04$, Mann Whitney U test)

Msh2 null mice exhibited a higher mutation frequency than wild type controls and *Msh2* heterozygous mice. *Msh2* null mice showed a marked increase in mutation frequency from 4 to 8 weeks: a period where there is a significant increase in the number of crypts (Shoemaker *et al*., 1995). Surprisingly, beyond this time point there was no further increase in mutation frequency. This may reflect selection for mice with a reduced predisposition to neoplasia (and potentially reduced mutation frequencies) at the later time points as the peak incidence of neoplasia occurs at 3–4 months.

The most intriguing result is the increase in mutation frequency in the heterozygotes aged over 12 months. Similar results have been shown using very sensitive mutation screens in *Msh2* and *Mlh1* heterozygous diploid yeast (Shcherbakova and Kunkel, 1999; Drotschmann *et al*., 1999, 2000). The authors of these studies argued that this increased mutation frequency occurred due to loss of heterozygosity in a small population of the heterozygote cells. However, although LOH at the *Msh2* locus cannot be formally ruled out as the mechanism underlying the increase in the aged hemizygotes, we believe this to be unlikely as this requires two mutations (inactivation of the remaining *Msh2* allele and subsequent inactivation of *Dlb-1b*) to occur sequentially within the same stem cell. Wild type cells accumulate on average one additional mutation per 10 000 crypts each month (Winton *et al*., 1988). If we assume a null hypothesis and that heterozygosity for *Msh2* plays no direct role in suppressing mutation, and furthermore that *Msh2* is inactivated at a similar rate to *Dlb1*, then after 13 months there would be approximately 13 *Msh2* null clones per 10 000 crypts. Our analysis shows *Msh2* deficiency increases the mutation frequency to, at maximum, less than 10 times that found in wild type cells (the highest fold difference of 9.6 was observed at 1 month). This indicates that the additional mutation burden from the *Msh2* null clones would be less than 1 mutation per 10 000 villi. This increase is clearly insufficient to account for the actual observed increase, strongly implying that the elevation in mutation frequency is occurring as a direct consequence of *Msh2* heterozygosity.

We interpret the effect of heterozygosity at the *Msh2* locus as leading to a constant but small increase in mutability which only becomes evident over a long period of elapsed time (in this instance 12 months of age). However, it remains formally possible that heterozygosity for *Msh2* may result in the mice becoming hypermutable at old age, a hypothesis which we have not addressed in this study, but one which could be addressed by future experiments.

using *Mlh1* heterozygous mice following MNU (N-methyl-N-nitrosourea) exposure have revealed increased rates of tumourigenesis compared to wild type controls (Kawate *et al*., 2000). We therefore investigated mutation frequency at the *Dlb-1* locus in young (8–12 weeks old) *Msh2* mutant mice following treatment with either MNNG (Sigma) or cisplatin (David Bull Laboratories) (Figure 3). Mutation was scored 3 weeks post i.p. injection. Following exposure to 50 mg/kg MNNG we observed a significant increase in mutation frequency in *Msh2* null mice compared to controls (Mann Whitney U test, $P=0.0414$), but not in heterozygotes (Mann Whitney U test, $P=0.17$). These results parallel those obtained following exposure to the alkylating agent Temozolomide (Toft *et al*., 1999), confirming that Msh2 normally suppresses mutation following damage of alkylation type but also showing that this response is not *Msh2* gene-dose sensitive. Following cisplatin exposure *Msh2* nulls showed increased mutation frequencies relative to controls and heterozygous mice, however these increases could entirely be accounted for by the increase in spontaneous mutation frequency. These results therefore fail to demonstrate a role for *Msh2* in mutation surveillance following cisplatin treatment and question the significance of mismatch repair in the clearance of cisplatin-induced DNA damage in normal cells. Our findings complement recent studies by Branch *et al*. (2000), who demonstrated that loss of MMR was only a minor contributor to cisplatin resistance in ovarian tumour cell lines.

The studies reported here have been performed in normal intestinal enterocytes. It remains possible that the reliance upon functional MMR for mutation surveillance markedly differs between normal and neoplastic cells. Pertinently, Strathdee *et al*. (2001) have shown that mouse embryonic fibroblasts deficient for *Msh2* still undergo a G2 arrest following cisplatin treatment in marked contrast to MMR deficient tumour cell lines which lose this checkpoint (Brown *et al*., 1997).

In conclusion, we demonstrate an extremely complex reliance upon *Msh2* in mutation surveillance. We show that *Msh2* is critical in monitoring spontaneous levels of DNA damage, such that even a 50% reduction in *Msh2* gene dosage can elevate mutation frequency. We show that this reliance upon *Msh2* in response to spontaneous levels of DNA damage is not directly reflected by gene dependencies in either the apoptotic response or mutation burden following acute DNA damage. We show that *Msh2* deficiency leads to an increase in the *in vivo* mutation frequency following MNNG treatment, but remarkably that this is not the case following exposure to cisplatin. Taken in the context of the recent proliferation of data relating to MMR-dependent DNA damage responses, these observations show that reliance upon functional MMR is highly lesion type dependent. They also challenge our understanding of MMR-dependent suppression of mutation, perhaps partly because MMR dependent responses are usually interpreted in an experimental setting following high, non physiological levels of DNA damage. Clearly, although such scenarios may directly relate to the response to chemotherapeutics in a clinical setting, they may not accurately reflect the reliance upon MMR in normal tissues exposed to low levels of spontaneous damage.

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Minireview

P53 null mice: damaging the hypothesis?

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Abstract

P53 is extremely well characterised as a tumour suppressor gene, and many activities have been attributed to it which are consistent with this function. However, despite being the subject of intense study it still remains unclear precisely which of these functions is crucial to its in vivo role as a tumour suppressor gene. This is particularly true of its role in the induction of apoptosis. The original observation of p53-dependent apoptosis gave rise to the following hypothesis: namely, that p53 deficiency leads to a persistence of DNA damaged cells which are the potential founders of malignancy. This review summarises the data for and against this hypothesis, with specific emphasis on data obtained from studies of the murine intestine. What emerges from these studies is a complex picture, where data can be obtained in support of this hypothesis, but there are many circumstances which exist where it is not supported. Taken together this collection of data suggests that the abrogation of p53-dependent apoptosis may indeed impact upon carcinogenesis and neoplastic progression, but that the simplistic notion of p53 as the single gatekeeper of this pathway is untenable.

Keywords: P53; Mice; Tumour suppressor gene

1. Introduction

Any search on p53 soon yields a staggering number of publications (in 1998 alone there were 3744 publications), highlighting that this is probably the most studied protein in scientific world. This statistic reflects the fact that p53 mutations are amongst the commonest molecular events in neoplasia: it is mutated in approximately 50% of human cancers and is possibly inactivated in all tumours through loss or upregulation of other genes in the p53 pathway, e.g. $p14^{ARF}$ and *Mdm2* [1,2].

For many years it has been known that p53 is a critical cellular mediator of the response to genotoxic damage in vivo and in vitro [3]. P53 has been shown to induce apoptosis (programmed cell death) in response to a wide variety of insults and shown in vitro to also induce G1 and G2 cell cycle arrest via transcriptional activation of p21/WAF [4].

Recently, a series of significant advances have been made in the in vitro characterisation of the p53 pathway. These include the identification of ever increasing types of cellular stress that upregulate p53; the identification of post-translational modifications in response to such stress, and the characterisation of proteins that interact with or are transactivated by p53 (see Fig. 1) (for a recent review see [5]). One obvious goal of these studies is to link stimuli, such as ionising

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vivo phenomenon, and even that it may be an artefact of the culturing procedure [8]. This represents an extreme interpretation of these difficulties, however, it serves to highlight the inherent difficulties in the study of a range of biological phenomena in vivo. Certainly the scope of in vivo analysis is currently restricted by the complexity of the in vivo setting and by the relatively limited availability of good in vivo tools.

This review will focus upon studies performed in vivo using p53 null mice and specifically on those experiments aimed at addressing the significance of p53 in inducing apoptosis following DNA damage. The observation that p53 deficiency abrogates the normal apoptotic response to DNA damage gave rise to the notion that the normal ability to engage apoptosis may underlie the role of p53 as a tumour suppressor. Hence, in the absence of functional p53 it was proposed that cells bearing DNA damage would persist, and that these 'undead' cells would carry an increased mutation burden, and therefore, be more likely to act as the founders of neoplastic clones [3]. Results addressing this hypothesis obtained from p53 null mice will be discussed and compared to similar data obtained from mice deficient in the mismatch repair gene *Msh2* (MutS homologue 2).

2. The phenotype of the p53-deficient mice

Three different strategies were employed to generate the first p53 knockout mice in the early 1990s [9–11]. All gave similar phenotypes with the majority of the p53 null mice dying from lymphoma at around 6 months. This gave definitive proof that p53 was a tumour suppressor gene, which previous clinical data and in vitro work had suggested [12].

Mice heterozygous for p53 can be viewed as a relatively accurate model of the Li-Fraumeni syndrome, as they develop tumours of a similar spectrum as those seen in Li-Fraumeni patients (oestosarcomas, lymphomas and soft tissue sarcomas) [13,14]. There are, however, two notable exceptions to this, namely, the high incidence of lymphoma in the mice and the absence of breast tumours (for recent reviews of mouse data see [15,16]). P53 heterozygotes develop symptoms of disease later than their p53 null counterparts, with approximately 50% of mice succumbing to tumours by 18 months. As wild type mice live for up to 36 months, this age of death of the heterozygotes is often compared to Li-Fraumeni patients where affected individual have a 50% chance of developing cancer by the age of 30 [17]. However, great care should be taken in making direct comparisons between the consequences of p53 deficiency in man and mouse as many species specific differences exist, perhaps most notably in the strong predisposition to lymphoma observed in the mouse.

Within the mouse, strain dependent differences in phenotypes have readily been observed [18,19]. For example, pure bred 129SV p53 null mice die from tumours much earlier than those crossed onto a C57Bl6 background. Furthermore, 50% of p53 null males in 129SV develop testicular teratomas which are only observed rarely in other backgrounds. This latter observation probably reflects that p53 deficiency accentuates any natural tumour predisposition, as wild type 129SV mice show a spontaneous predisposition to testicular teratomas compared to other strains [19–21].

3. P53 and development

Prior to the development of mice deficient for p53, a number of different in vitro studies had suggested key roles for p53 in initiating both apoptosis and cell cycle arrest. These predicted a severe phenotype for p53 deficiency, and it was therefore, of some surprise that p53 deficiency did not result in embryonic lethality as had been reported for other cell cycle genes, such as Retinoblastoma (*Rb*) [22]. This led to the suggestion that p53's true role was as a 'guardian of the genome', with a primary function of protecting against genotoxic damage [23]. Any role for p53 in development was therefore initially discounted, however, several lines of evidence have now made it clear that p53 does indeed play a role in normal embryonic development.

A common misconception when trying to understand the function of tumour suppressor genes is to presuppose that their sole function lies in the prevention of malignancy. However, if this were the case it is difficult to understand the common inheritance of tumour suppressor genes across many organisms that are clearly not prone to malignancy. Moreover, it is difficult to identify the selection pressure for retention of such a class of genes if the phenotype of cancer is only manifested post-reproduction [24]. These points

approach to overexpress a Mdm2 transgene and so cause loss of p53 [36].

4. Measuring mutation frequency in vivo

The fact that the majority of p53 null mice (and *Msh2* null) are viable has allowed questions to be posed addressing the importance of loss of gene function for phenotypically normal cells in vivo, when previously these questions had been restricted to (usually immortalised) cell lines in vitro. Although studies on such lines have produced invaluable data and will continue to do so, nearly all of these lines carry multiple genetic alterations. This makes the task of dissecting out the effect of individual genes very difficult.

The advent of knockout technology has not only produced a range of new in vivo systems, but it has also permitted the production of a new series of in vitro models. An obligatory step in the production of the knockout mice is the generation of targeted embryonic stem (ES) cells. If so desired, these can be rendered homozygous by either a second round of targeting or by the use of high levels of the appropriate antibiotic, which can select for conversion of the wild type allele. The availability of mutant cell lines are not, however, restricted to ES cells, as primary lines, such as embryonic fibroblasts (MEFs) can relatively easily be derived from mutant strains. Such primary lines carry the considerable advantage that they will not have been selected for additional genetic change in culture, and so should parallel the genotype of the mutant strain.

In each of these systems attempts have been made to determine the effect of genotype upon mutation rate. In reality these assays measure mutation frequencies, and broadly fit into two categories. First, those that that focus on a phenotypic change due to inactivation or overexpression of a somatic gene which is either autosomal or X-linked. These include changes resulting in loss of function at the *Dlb1* [37] and hypoxanthine phosphoribostltransferase (*HPRT*) loci [38], overexpression of metallothinein [39] and ouabain resistance [40]. The second category includes those approaches which measure mutation frequency from exogenous transgenes, including *lacI* (Big Blue^{TM}, *lacZ* (Muta^{TM}) and *supF* (tRNA suppressor gene) [40].

The most commonly used in vivo assays in the mouse are the *Dlb1* assay, and assays using the *lacI* and *lacZ* transgenes [41,42]. The *HPRT* is the most commonly used assay for in vitro estimates [38]. All of these techniques have drawbacks associated with them, however, it is encouraging that many of the in vivo tests yield relatively similar frequencies (see [43]).

The *Dlb-1* locus encodes for 2 lectin binding proteins. *Dlb-1a* is constitutively expressed on vascular endothelium. The *Dlb-1b* allele specifies binding of the Dolichos biflorus agglutinin to intestinal epithelium. Thus, in mice heterozygous for these alleles a single inactivating mutation at the *Dlb-1b* will abrogate the ability to bind this lectin in the intestinal epithelium. After cell proliferation and clonal expansion, mutations that occurred in the stem cell population will form clones that fail to bind a peroxidase conjugate of Dolichos biflorus agglutinin. These mutant clones can easily be visualised and scored to give an estimate of the mutation frequency.

As with the other approaches, the *Dlb-1* assay does suffer from some shortcomings. One drawback of this approach is that it is limited to the analysis of intestinal mutation rates, as specific *Dlb1-1b* expression is restricted to the intestine, and thus, mutation frequency can only be estimated in this tissue. A further difficulty is that the locus has not yet been cloned and so questions relating to mutational spectra cannot be addressed [44]. The assay does, however, possess a number of unique advantages over other approaches. Key amongst these is its ability to specifically score mutation within the stem cell population and to permit visualisation of the mutant clones in situ [41]. Furthermore, because the *Dlb-1* locus is an endogenous allele, there are few concerns over the in vivo relevance of results, as have been raised following the use of an exogenous transgene.

When mutation frequencies are compared between the *Dlb1* assay and the transgenic mice Big Blue^{TM} and MutA^{TM}, the transgenic models generally appear a little less sensitive [43]. You et al. [45] showed that one of the reasons for this was the presence of a large number of CpG sites in the *lacI* gene which are methylated in vivo. The *lacI* transgene usually consists of multiple concatemers (around 40) of the *lacI* gene within a λ-like shuttle vector. This can be harvested from the mouse genome and packaged within the λ phage. Bacteria are then infected on XGAL containing plates. After lysis those phages which contained

Second, the spectrum of mutations identified by these assays may be inappropriate for the type of instability produced by lack of p53. However, this possibility seems extremely unlikely given the concordance between the different types of assay.

A third possibility arises from the fact that a large proportion of p53 mutations in human tumours are dominant negative, implying that the nullizygous state may be an inappropriate model for the study of p53 as a tumour suppressor [12]. However, the vast majority of studies strongly argue that loss of function of p53 is functionally equivalent, whether through the acquisition of a dominant negative mutation or through a homozygous null genotype. Although this difference is an important caveat to remember when considering the p53 null mouse as a model for p53 mediated tumourigenesis, it is extremely unlikely to bear any significance on the role played by p53 in protecting against mutation in normal cells.

A fourth possibility is that p53 is only relevant in guarding against increased mutation within abnormal or malignant cells. However, this also seems unlikely, as when Buettner et al. [53] analysed thymic lymphomas from p53 null mice only one out of four tumours had an increased mutation frequency compared to normal thymus. In four tumours examined by Sands et al. [52], none were found to have an increased mutation frequency. This in itself is a fascinating observation, questioning the mutator hypothesis of cancer which attempts to explain why tumours acquire so many somatic mutations (far above the postulated mutation rate for somatic tissues) (see [54]).

Finally, it may be that p53 cannot be shown to play a role because it is essentially redundant. Recently, several p53 homologues have been identified that could theoretically substitute for p53 in the p53 nulls. For example, the homologue p73 has been implicated in p53 independent apoptosis following both cisplatin treatment and ionising radiation [55,56]. Obviously, this cannot reflect complete redundancy, otherwise no exclusively p53-dependent phenomena (such as p53-dependent apoptosis) would be observed. However, given that mutation frequency is almost certainly determined by a number of interacting factors, redundancy remains a tenable explanation.

Given the current state of the field, it is not yet possible to discriminate between these possibilities. However, the fundamental observation that deficiency of p53 fails to influence spontaneous mutation rate has challenged the view that p53 plays a direct role that in DNA repair. It has also questioned the in vivo significance of p53 dependent apoptosis and cell cycle arrest.

6. Apoptosis, clonogenic survival and mutation frequency in vivo

The preceding discussion has addressed the consequences of loss of gene function at spontaneous levels of DNA damage. These studies are clearly limited to the low levels of environmental insult that may exist in a normal laboratory animal house setting, and will therefore, not address the consequences of exposure to defined types of DNA damage. Analysis using spontaneous levels of DNA damage also precludes the determination of a series of endpoints. Thus, apoptotic dependency can only be scored in circumstances that induce apoptosis. Similarly, clonogenic survival can only be scored when the majority of cells are lethally damaged. This is a particularly crucial endpoint to study as the emergence of malignant clones must be absolutely dependent upon the long term survival of the founder cell. For these reasons mice mutant for p53 and *Msh2* have been studied following exposure to defined types of DNA damage.

Amongst the first studies performed using the p53 knockout mice were those investigating the p53-dependency of apoptosis following genotoxic insult (normally ionising radiation). Initially Clarke et al. [57] and Lowe et al. [58] showed that unlike wild type thymocytes, which rapidly undergo apoptosis following treatment with ionising radiation and etoposide, p53 null thymocytes were resistant. Heterozygotes were found to have an intermediate phenotype. However, p53 null thymocytes were not resistant to the glucocorticoid methylprednisoline, nor to apoptosis induced following treatment with a calcium ionophore. These experiments showed that p53-dependence was restricted to certain types of stress, usually clastogenic damage. These observations gave rise to the notion that the failure to engage apoptosis may be the critical predisposing factor to tumourigenesis in a p53 null environment. Thus, cells exposed to DNA damage would not be deleted in the absence of p53, but would persist with a higher mutation burden, and therefore, a greater predisposition to malignancy.

difference between p53 nulls and wild types. However, p53-dependent difference was observed at the higher dose of 600 rads. Notably, this increase was not seen to be p53 gene-dose dependent, despite the fact that heterozygotes have an intermediate apoptotic phenotype.

One possible explanation for these results is that p53-independent apoptosis may be sufficient to remove cells harboring DNA damage cells following exposure to low doses of ionising radiation, but that this mechanism is incapable of dealing with the damage inflicted at high doses. Such an explanation does not, however, rest easily with the failure to see a p53-dependent difference in clonogenic survival, as this predicts a difference at the high levels of DNA damage used in the reported assays.

It is also quite possible that the nature of p53-dependency will alter with other insults. For example, we have obtained data indicating a clear p53-dependency on clonogenic survival following treatment with cisplatin [64].

In summary, the available data examining the relationship between apoptosis, clonogenic survival and mutation frequency in vivo is at the least inconsistent with the simple hypothesis originally proposed. Indeed, the clearest published demonstration of p53 dependency in clonogenic survival appears to disassociate apoptosis and clonogenicity [70]. Here, both low (40 mg/kg) and high (400 mg/kg) doses of the drug 5 fluorauracil (FU) was used. Both induced similar levels of apoptosis, which was p53 dependent at the 24 h peak (although in the wild types the levels of apoptosis returned back to basal levels sooner in the animals treated at 40 mg/kg). However, it was only the mice treated at the higher dose which showed increased p53-dependent clonogenic survival. Pritchard et al. [70] suggested that this was due to changes in cellular proliferation as mitotic cell indices and thymidine incoroparation fell to a much lower level in the mice treated at the higher dose. Thus, the presence or absence of a p53 dependent growth arrest is suggested as the crucial factor governing clonogenic survival. These results again strongly suggest that simple correlations cannot readily be drawn between loss of apoptosis, increased clonogenic survival and mutation frequency.

The situation as described for p53 contrasts somewhat with studies performed on *Msh2* deficient mice treated with the alkylating agents temozolomide and MNU (*n*-methyl-*n*-nitrosourea) [50,51]. Both of these drugs produce the cytotoxic O^6 methylguanine lesion. This lesion generally produces a G–T mispair after replication which is recognised by the mismatch repair machinery. Karran and Bignami [71] proposed that as this is recognised as a mismatch, the MMR machinery will remove the newly synthesised T. However, there will be no base that will correctly pair with the O^6 methylguanine lesion so it is proposed that this causes cycles of abortive repair which will eventually lead to apoptosis [71]. Toft et al. [51], showed that in the small intestine, *Msh2* deficiency reduces the apoptotic response to both temozolomide and MNNG (5 methyl-*N'*-nitro-*N*-nitroguanidine). Unlike the situation with p53 deficiency, there was no delayed *Msh2*-independent death. When clonogenic survival and mutation frequency was investigated in vitro in *Msh2* null ES cells, these showed both an increase in survival and mutation frequency relative to wild type controls. Significantly, an element of the increase in mutation frequency could be attributed to increased clonogenic survival. When mutation frequency was measured in vivo at the *Dlb-1* locus, there was a significant dose dependent increase in mutation after temozolomide treatment. Andrew et al. [50] also showed a significant increase (above basal levels) in mutation using the Big Blue™ mouse after treatment with MNU (*N*-methyl-nitrosourea). Thus, here there seems to be a clear relationship between loss of apoptosis and increase in survival and mutant cells, indicating that loss of apoptosis could be important in predisposing to malignancy. However, it is worth noting that as with many studies of this type, the level of DNA damage used was far from physiological. This may be of particular relevance when studying alkylating agents, as many O^6 methylguanine lesions are normally removed in vivo by the action of ATase (O^6 alkylguanine transferase). It may be that defects in the mismatch repair machinery only become relevant when this pathway is saturated, for example as might occur following high dose chemotherapy [72].

8. P53 and tumourigenesis

From the above it is possible to conclude that although p53 is clearly important in inducing apoptosis

response to DNA damage, as predicted from in vitro studies. However, experiments using the p53 null mice have identified a number of surprising results. Perhaps the first of these was that the null animals survived to birth at all, given the widespread roles proposed for p53. A partial role for p53 in embryogenesis has now been established. Other results have directly contradicted predictions from experimental systems. After genotoxic insult, despite the presence of a wave of p53-dependent apoptosis, increases in clonogenic survival and mutation are only seen in some systems and in some circumstances. Thus, for both of these phenomena there are tissue and drug specific variations in p53-dependency, and the significance to neoplasia of p53-dependent in vivo apoptosis remains to be proven.

These findings contrast somewhat to those obtained from mice deficient in the mismatch repair protein *Msh2*, which show clear increases in mutation following exposure to alkylating agents. ES cells mutant for *Msh2* also show increased clonogenic capacity, at least when scored in vitro. These increases are consistent with the observed loss of an *Msh2*-dependent apoptotic pathway, although the relative importance played by *Msh2*-dependent repair and *Msh2*-dependent apoptosis remains to be established in vivo [51].

It is also now clear that p53-independent pathways exist which can mediate the delayed induction of apoptosis in the absence of p53, and that these may be mediated by p53 homologues. It may be that only when these other pathways are fully characterised will we be able to determine the true relevance of cell death to neoplasia. For example, we have been able to show that loss of both *Msh2* and p53 causes complete loss of the apoptotic response following treatment with temozolomide [51]. This observation predicts a number of phenomena that can be directly tested, for example that treatment of an *Msh2* null tumour with an alkylating agent, such as temozolomide will select for loss of p53 (and vice versa).

In summary, the generation of p53 knockout mice has yielded a vast body of data on p53 function in vivo, and yet it may have left us with many more questions than answers. The original supposition that tumour suppression would be simply mediated by the apoptotic deletion of DNA damaged cells now seems somewhat naive. We, therefore, remain faced with the task of unravelling the in vivo relevance of each different aspect of tumour suppressor activity, both for p53 and its related genes. This process will be greatly facilitated by the rapid advancement in our understanding of the molecular pathway of p53 in vitro and by the generation of more advanced transgenic models.

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In vivo administration of O^6-benzylguanine does not influence apoptosis or mutation frequency following DNA damage in the murine intestine, but does inhibit P450-dependent activation of dacarbazine

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Clinically relevant cancer chemotherapeutic alkylating agents such as temozolomide and dacarbazine induce apoptosis and are mutagenic via the formation of O^6-alkylguanine adducts in DNA. The DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) functions by dealkylating such adducts and can thus prevent apoptosis and mutagenesis. In attempts to maximize the clinical effectiveness of these alkylating agents, inhibitors of AGT such as O^6-benzylguanine (BeG) have been developed. We show here that within murine small intestinal crypt cells, BeG administration does not alter the apoptotic response to the direct-acting methylating agents *N*-methyl-*N*-nitrosurea (MNU), temozolomide and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Furthermore, we show that BeG pretreatment fails to elevate the mutation frequency at the murine *Dlb-1* locus following exposure to MNU. Consistent with these results, we show that intestinal AGT activity is effectively abolished by administration of 100 mg/kg temozolomide, even in the absence of BeG. In contrast, pretreatment with BeG transiently abolished the apoptotic response to the methylating prodrug dacarbazine. Activation of dacarbazine to its reactive intermediate has previously been shown to be cytochrome P450 dependent and we show here that pretreatment of mice with the cytochrome P450 inhibitor metyrapone also inhibits dacarbazine-induced apoptosis. Thus BeG increases neither the prevalence of apoptosis nor mutation frequency in the murine small intestine, but is capable of inhibiting P450-dependent prodrug activation. The positive implication from this study is that BeG treatment may not exacerbate the toxic and mutagenic effects of methylating agents within normal cells, although it may engender other adverse reactions through the suppression of cytochrome P450-dependent processes.

Introduction

The murine small intestine offers an ideal experimental system to study both apoptosis and mutation frequency in response to DNA damaging agents such as γ-irradiation and alkylating agents (1–3). The DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) is expressed in the murine small intestine and is up-regulated in a *p53*-dependent manner in response to DNA strand breaks following ionizing irradiation (4). AGT functions by recognizing and removing specific alkyl lesions from DNA (5). The principal substrate is O^6-methylguanine, which is the major toxic and premutagenic lesion induced by methylating agents. AGT-mediated repair occurs via transfer of the alkyl group to a cysteine residue in the AGT protein, a process which is stoichiometric and autoinactivating (6). AGT therefore confers protection against the mutagenic and toxic effects of alkylating agents, which include the clinically important antitumour agents temozolomide and 5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide (dacarbazine) (7–10). Failure of AGT to repair O^6-methylguanine results in O^6-methylguanine:thymine mispairs following DNA replication and it is postulated that these mediate cell death via mismatch repair (11). The DNA mismatch repair enzyme MSH2 is known to bind to and recognize O^6-methylguanine:thymine mispairs and is also essential for a large proportion of apoptosis observed in the murine small intestine following exposure to alkylating agents (12,13). Mutations arise if the O^6-methylguanine:thymine mispairs undergo further rounds of replication.

O^6-benzylguanine (BeG) is a competitive and irreversible inhibitor of AGT which acts by binding to the $-CH_3$ cysteine acceptor site of AGT forming *S*-benzylcysteine (14,15). BeG-bound AGT is inactive and is subsequently degraded. Cellular AGT activity can be restored only by *de novo* protein synthesis (5) and its depletion by BeG has been proposed as a useful adjuvant to clinical methylating and chloroethylating agent treatment of tumours (16). Adjuvant therapy of BeG combined with such agents offers the potential to use lower, and hence less toxic, doses of chemotherapeutic drug and may also be of benefit in overcoming tumour resistance associated with high levels of AGT (17). Dacarbazine is a DNA alkylating agent used in the treatment of metastatic melanoma and Hodgkin's lymphoma (18). It is a prodrug requiring cytochrome P450-dependent *N*-demethylation to produce the active compound 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MITC) (19,20). MITC is also generated when temozlomide reacts spontaneously with H_2O (21). MITC decomposes to a methylating intermediate common to a variety of agents that result in the methylation of DNA with subsequent consequences for cell death and mutation.

One approach to directly determine the somatic *in vivo* mutation frequency relies upon detecting loss of function at the polymorphic genetic locus *Dlb-1* (22). This locus determines the ability to bind the lectin from *Dolichos biflorus* in mouse intestinal epithelium. Mutation frequency can be scored by identifying clonal populations which are no longer capable of binding the lectin. We have previously shown that *in vivo* administration of BeG reversibly suppresses AGT function, but does not modulate the apoptotic response to temozolomide

Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; BeG, O^6-benzylguanine; DBA, *Dolichos biflorus* agglutinin; dacarbazine, 5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide; MITC, 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosurea; PBS, phosphate-buffered saline.

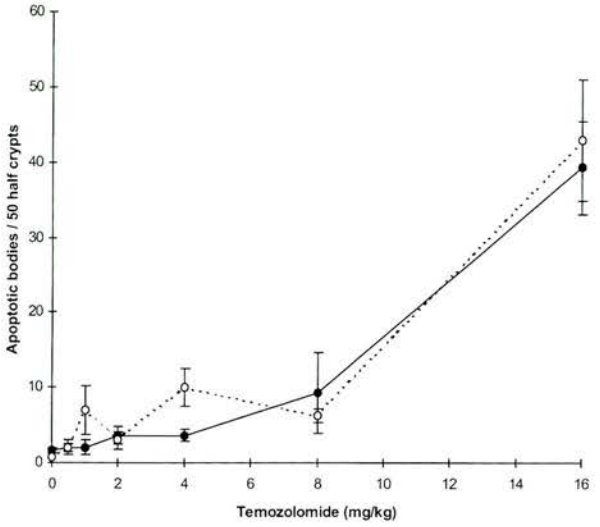

Fig. 2. The effect of BeG upon apoptosis induced by varying doses of temozolomide. Cohorts of wild-type mice treated with temozolomide (range 0.5–16 mg/kg) in the presence and absence of BeG. Data show the prevalence of apoptosis 6 h after temozolomide administration and 7 h after i.p. injection of BeG (60 mg/kg). Each point represents data from a minimum of three mice. Closed circles, BeG treated; open circles, no BeG. Error bars represent SEM.

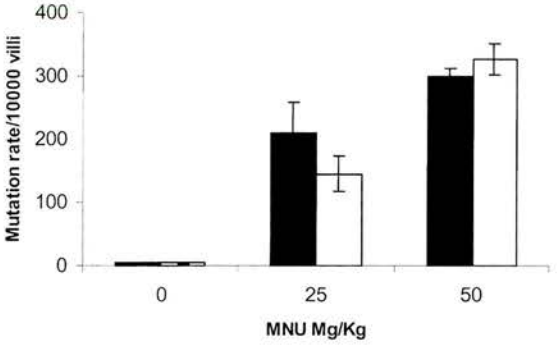

Fig. 3. The effect of BeG on mutation rates following exposure to MNU. *Dlb-1* mutation frequency in the murine small intestine following 100 mg/kg MNU treatment in the presence and absence of BeG. BeG was administered at a dose of 60 mg/kg prior to the administration of MNU. Each point represents data from three mice. Filled bars, no BeG; open bars, BeG treated. Error bars represent SEM.

and therefore rendering BeG treatment ineffective, very low doses of temozolomide (range 0.5–16 mg/kg) were administered to mice with and without BeG administration. Apoptosis was scored 6 h later (Figure 2). The rationale was that at low doses of temozolomide not all functional AGT activity would be abolished by the alkylating agent and thus, if AGT could actually alter the level of apoptosis, a difference between BeG-treated and non-treated mice may be revealed. No significant differences in the incidence of apoptosis were observed between BeG-treated and non-treated mice at any dose of temozolomide ($P > 0.07$ for all doses, Mann–Whitney *U*-test).

MNU-induced mutation frequency at the Dlb-1 locus is not influenced by BeG

Mutation rate within the murine small intestine following MNU was scored at the *Dlb-1* locus using standard approaches (1,22). The mutation frequency increased with increasing doses of MNU (Figure 3), but prior treatment with BeG did not result in a significant elevation in the mutation frequency at the *Dlb-1* locus ($P > 0.05$ for both doses, Mann–Whitney *U*-test).

Temozolomide administration effectively ablates AGT activity in the intestine

One explanation for the failure to observe BeG-dependent differences in apoptosis and mutation frequency is that treatment with the methylating agent alone is sufficient to render AGT functionally inactive and that, further, BeG-mediated suppression is therefore largely irrelevant. To directly test this we exposed mice to varying doses of temozolomide and scored AGT activity in both the liver and intestine. As we have shown before, pretreatment with BeG markedly reduces resting levels of AGT activity at 6 h (13). In the liver this reduction is from 150 ± 40.8 to 13.3 ± 21 fmol/mg protein. In the intestine AGT activity falls from a lower resting level (45.6 ± 12 fmol/mg) to become undetectable 6 h after BeG treatment (13). The effect of exposure to increasing doses of temozolomide was indeed to reduce levels of AGT activity, as shown in Figure 4. Following exposure to 100 mg/kg temozolomide, AGT activity was effectively depleted in both the liver and small intestine.

BeG inhibits the P450-dependent metabolic activation of dacarbazine

We have shown that exposure to BeG inhibits apoptosis induced by dacarbazine (Figure 1). In order to study the kinetics of this inhibition a single dose of BeG (60 mg/kg) was administered to wild-type mice at time 0. Mice were then subsequently injected with 150 mg/kg dacarbazine at 0, 1, 6, 12, 24, 48 and 72 h after BeG delivery. Six hours after each dacarbazine injection, levels of apoptosis were scored in the small intestine (Figure 5). Dacarbazine alone induced high levels of apoptosis with a mean of >60 apoptotic bodies per 50 half-crypts. However, between 1 and 6 h following BeG administration dacarbazine failed to induce apoptosis. This suppression was reversible, such that a normal apoptotic response was restored by 24 h. Thus, BeG administration reversibly inhibited dacarbazine-dependent apoptosis. The active DNA-damaging metabolite of dacarbazine is MITC, which is generated from dacarbazine in a cytochrome P450-dependent manner. In order to confirm that cytochrome P450 function was essential in eliciting a dacarbazine-dependent apoptotic response we used the P450 inhibitor metyrapone (25). Dacarbazine was administered 1 h following treatment with metyrapone (100 mg/kg i.p.). Metyrapone treatment significantly reduced the apoptotic response to dacarbazine (Figure 6), confirming that activation of the prodrug and the associated apoptotic response were P450 dependent ($P < 0.05$, Mann–Whitney *U*-test). These findings indicate that BeG administration blocks activation of the prodrug dacarbazine, possibly by compromising host P450 activity.

Discussion

We have directly addressed the ability of BeG to alter rates of apoptosis and mutation in the normal murine epithelium following exposure to a range of different types of damage. No induction of apoptosis was associated with BeG treatment. This excluded the possibility that either BeG itself or the depletion of AGT activity could result in the induction of apoptosis in the murine small intestine. Following treatment with BeG, and in the absence of detectable AGT activity, exposure to MNU, cisplatin and γ-irradiation all induced

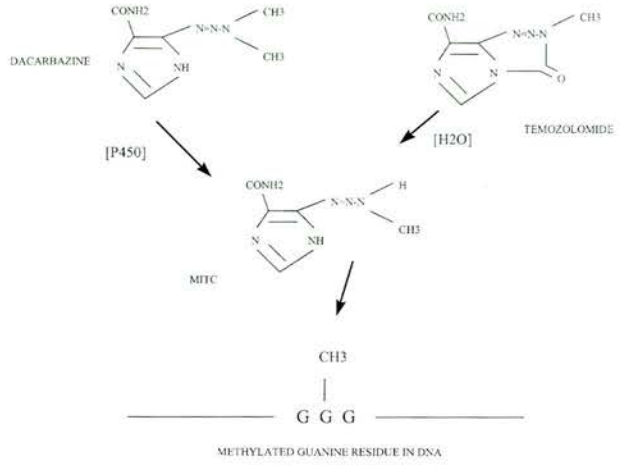

Fig. 7. Degradation pathways for dacarbazine and temozolomide. Dacarbazine and temozolomide give rise to the active compound MITC, which decomposes spontaneously to an active methylating agent which produces, amongst 11 other base lesions, O^6-methylguanine, the major premutagenic lesion in DNA. Dacarbazine requires oxidative *N*-demethylation by cytochrome P450 to form MITC, unlike temozolomide, which reacts spontaneously with water to give MITC.

to an active alkylating agent. Dacarbazine is known to undergo oxidative *N*-demethylation to its active compound MITC (18,28,29). This metabolic activation is mediated through the action of cytochrome P450 enzymes (20,30). Two observations argue in favour of the possibility that BeG is blocking the normal metabolism of dacarbazine. First, temozolomide is able to induce apoptosis in the presence of AGT depletion (13). Thus, the inhibitory action of BeG cannot be arising subsequent to the generation of MITC since it is also the active compound formed when temozolomide undergoes spontaneous chemical transformation (19,21) (Figure 7). Second, one of the major routes of metabolism of BeG in mice involves oxidation to form O^6-benzyl-8-oxoguanine (31), which occurs through the actions of aldehyde oxidase and the cytochrome P450 isoforms CYP1A2 and CYP3A4 (32,33). Dacarbazine is also metabolized by a number of different isoforms of P450, including CYP1A1, CYP1A2 and CYP2E1 (34). For both dacarbazine and BeG, the primary isoform responsible for metabolism is CYP1A2. The possibility therefore arises that BeG metabolism may competitively deplete cytochrome P450 activity. Thus, BeG appears capable of inhibiting at least some of the demethylating ability of cytochrome P450 enzymes in the mouse liver. The consequences of this novel finding are likely to be significant in view of the central role of P450 enzymes in the metabolism of drugs and carcinogens.

There is increasing interest in the use of BeG clinically as an adjuvant to alkylating agent chemotherapy to overcome tumour resistance mediated by AGT and to potentiate the cytotoxic effects of chemotherapy (16,35). There are, however, a number of uncertainties concerning the clinical benefits of BeG. First, alkylating agents themselves are capable of inactivating AGT activity by depleting cellular pools (5,27). We show here that this can be an effective mechanism for suppressing AGT activity both in the normal cells of the small intestine and in the liver of mice. There are, however, several lines of evidence to suggest that the use of alkylating agents to suppress AGT activity can be ineffective or even inappropriate, either because of failure to increase the therapeutic index or because of dose-limiting toxicity (27). Thus, although alkylating agents can reduce the activity of AGT, the available data indicate BeG administration to be the most effective and least toxic method to achieve this. Second, BeG has been reported to increase mutation rates, chromosome aberrations and toxicity in normal cells treated with methylating or chloroethylating agents (36–38). In contradiction to these reports, we do not observe any effect upon *in vivo* mutation rate in these studies. Third, from the data presented here it is clear that in the murine small intestine AGT depletion does not elevate *in vivo* apoptotic levels following methylating DNA damage. This result has clear positive implications for the therapeutic use of BeG, in that BeG administration fails to augment cell death within normal tissues. This result may directly follow from the demonstrated ability of alkylating agents to partially suppress AGT activity and may simply reflect the low resting levels of AGT in the murine intestine. Finally, and perhaps most significantly, these data show that BeG can alter the metabolism of drugs or carcinogens, exemplified here by activation of the prodrug dacarbazine, and may therefore be a cause of unexpected adverse drug reactions if used clinically.

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SHORT REPORT

A role for mismatch repair in control of DNA ploidy following DNA damage

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Many reports have shown a link between mismatch repair (MMR) deficiency and loss of normal cell cycle control, particularly loss of G2 arrest. However almost all of these studies utilized transformed cell lines, and thus the involvement of other genes in this phenotype cannot be excluded. We have examined the effects of cisplatin treatment on primary embryo fibroblasts (MEFs) derived from mice in which the MMR gene Msh2 had been inactivated ($Msh2^{-/-}$). This analysis determined that both primary $Msh2^{-/-}$ and wild type (WT) fibroblasts exhibited an essentially identical G2 arrest following cisplatin treatment. Similarly, we observed a cisplatin-induced G2 arrest in immortalized MMR deficient ($Mlh1^{-/-}$ and $Pms2^{-/-}$) and WT MEFs. p53 deficient primary MEFs ($p53^{-/-}$) exhibited both a clear G2 arrest and an increase in cells with a DNA content of 8N in response to cisplatin. When the *Msh2* and *p53* defects were combined ($p53^{-/-}/Msh2^{-/-}$) the G2 arrest was essentially identical to the $p53^{-/-}$ fibroblasts. However, the $p53^{-/-}/Msh2^{-/-}$ fibroblasts demonstrated a further increase in cells with an 8N DNA content, above that seen in the $p53^{-/-}$ fibroblasts. These results suggest that loss of MMR on its own is not enough to overcome G2 arrest following exposure to cisplatin but does play a role in preventing polyploidization, or aberrant DNA reduplication, in the absence of functional p53. *Oncogene* (2001) **20,** 1923–1927.

Keywords: mismatch repair; cell cycle; polyploidy; p53; DNA damage

DNA mismatch repair is known to play an important role in maintaining genomic stability, due to its function in correcting DNA mismatches introduced during replication, and cells deficient in MMR exhibit an increased mutation rate (for review see Buermeyer *et al.*, 1999). However the proteins that make up the MMR system have also been associated with a number of other phenotypes. Cells deficient in MMR have been shown to be resistant to a number of clinically important drugs, such as cisplatin, temozolomide and doxorubicin (Fink *et al.*, 1998). This resistance may be mediated by an altered ability to engage apoptosis, as the MMR proteins have been shown to be critical in mediating the normal *in vivo* apoptotic response to alkylating agents (Toft *et al.*, 1999). Restoration of MMR activity in cell lines, either by chromosome transfer or by direct re-introduction of the gene, results in drug resensitization, demonstrating that the drug resistance is not secondary to an increase in mutation rate but is due to the direct involvement of the MMR system. In addition MMR deficiency has also been associated with a loss of normal cell cycle controls in response to agents such as MNNG (Koi *et al.*, 1994), 6-thioguanine (6-TG) (Hawn *et al.*, 1995) cisplatin (Brown *et al.*, 1997) and ionizing radiation (IR) (Davis *et al.*, 1998), suggesting that the MMR system may have a regulatory role, primarily at the G2/M boundary. Indeed the activity of a number of key regulators of the cell cycle, such as p53 (Duckett *et al.*, 1999), p73 (Gong *et al.*, 1999) and c-*abl* (Nehme *et al.*, 1997), are known to be modulated in a MMR dependent fashion in response to certain types of DNA damage.

Loss of MMR has also been shown to play an important role in the development of cancer. Loss of at least five of the MMR proteins (MLH1, MSH2, MSH6, PMS1 and PMS2) has been shown to be associated with the hereditary cancer syndrome HNPCC (Buermeyer *et al.*, 1999). In addition loss of mismatch repair, and the resulting microsatellite instability phenotype, is seen in a significant fraction of sporadic tumours of many different cancer types (Arzimanoglou *et al.*, 1998). It is generally assumed that loss of MMR leads to cancer due to the resulting increased mutation rate, although it has also been suggested that defective cell cycle control may be the primary oncogenic mechanism in MMR deficient cells (Tomlinson and Bodmer, 1999).

Nearly all studies on the role of MMR in drug resistance and cell cycle control have been performed in tumour-derived or immortalized cell lines. Such cell lines will inevitably have multiple alterations in cell cycle control, which may well interact with a deficiency in MMR and influence the apparent phenotype of MMR defects. To more clearly define the role of MMR in the cell cycle response to a commonly used chemotherapeutic agent, cisplatin, we have assessed cell

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Advances in Brief

Suppression of Intestinal and Mammary Neoplasia by Lifetime Administration of Aspirin in *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$, *Msh2*$^{-/-}$ Mice[1]

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Abstract

Numerous studies have indicated that exposure to nonsteroidal anti-inflammatory drugs is associated with a lowered risk of colorectal cancer. However, analyses of the effect of aspirin upon tumorigenesis in *Apc*$^{Min/+}$ mice have yielded contrasting results. We show that adult dietary exposure to aspirin does not suppress intestinal tumorigenesis in *Apc*$^{Min/+}$ mice, but that continual exposure from the point of conception does. To test whether this regime could suppress the phenotype of murine models of hereditary nonpolyposis colorectal carcinoma, *Msh2*-deficient mice were exposed to aspirin. This did not modify the mutator phenotype of *Msh2*$^{-/-}$ mice, but weakly extended survival. Finally, we analyzed (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) mice and found that lifetime aspirin exposure significantly delayed the onset of both intestinal and mammary neoplasia. Thus embryonic and perinatal exposure to aspirin suppresses neoplasia specifically associated with the loss of *Apc* function, opening a potential window of opportunity for nonsteroidal anti-inflammatory drug intervention.

Introduction

Substantial epidemiological evidence shows that treatment with NSAIDS[3] reduces the risk of developing colorectal cancer. The most commonly used NSAID has been aspirin, which has been reported to reduce the risk of colon cancer by up to 40%. Clinical studies using the NSAID sulindac have also reported reduced polyp counts in FAP patients (1, 2). The precise mechanism of NSAID action remains unclear, although the suppression of Cox2 is thought to be of pivotal importance. Significantly, Cox2 is overexpressed in ~85% of human colorectal adenocarcinomas and adenomas from the *Apc*^{Min} mouse (3). The definitive study highlighting the relevance of Cox2 to tumorigenesis showed that a Cox2-deficient background markedly suppressed intestinal neoplasia in mice carrying the *Apc*$^{\Delta 716}$ allele (4). The biological activities of aspirin and sulindac are not restricted to suppression of the Cox2 pathway. Aspirin, sulindac, and even the selective Cox2-inhibitor celecoxib (5) have been shown to induce apoptosis in a Cox2-independent fashion. Both aspirin and sulindac down-regulate β-catenin- and β-catenin/TCF4-mediated transcription (6, 7). NSAIDS also act on the nuclear factor κB signaling pathway (8), and recent data suggest this may be important for their antitumor activities (8). They have also been shown to specifically reduce the survival of genetically unstable (MSI+) MMR-deficient colorectal cancer cell lines (9), raising the possibility that aspirin may also suppress malignancy in hereditary nonpolyposis colorectal carcinoma families characterized by mutations in the *MMR* genes. Mice constitutively heterozygous for the *Apc*^{Min} allele have been used to determine the ability of the NSAIDs to suppress intestinal malignancy. However with respect to aspirin, these studies have produced contrasting results. Virtually every study [*e.g.*, Beazer-Barclay *et al.*, (10) has shown that sulindac causes a reduction in the number of the spontaneous malignancies, apart from Oshima *et al.* (4) who use relatively low levels of sulindac]. However, of the studies that have investigated spontaneous intestinal malignancy after aspirin treatment, only two have shown suppression of malignancy in the Apc^{Min} mouse. Two other studies failed to show suppression in either the *Apc*^{Min} mouse or the *Apc*^{1638N} mouse (6, 11–13). The basis for these discrepancies may lie within differences in the aspirin regime used. Shoemaker *et al.* (14) and Reitmair *et al.* (15) have argued that the majority of adenomas are fixed either *in utero* or perinatally just after birth.

Here we directly test the effect of increasing the period of aspirin exposure to include the entire period from the point of conception onwards. Furthermore, we have investigated whether this regime can modify the development of MMR-associated neoplasia by investigating the course of neoplasia in cohorts of *Msh2*$^{-/-}$ and (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) mice. We find that prolonged aspirin exposure dramatically enhances the suppression of *Apc*-associated neoplasia both within the intestine and the mammary gland but only weakly influences the phenotype of MMR deficiency.

Materials and Methods

Administration of Dietary Aspirin at Weaning. C57BL/6 mice wild-type and heterozygous for the *Apc*^{Min} mutation were placed on diets containing either 0, 200, or 400 mg/kg of aspirin (Harlan/Tekad). These levels of exposure are comparable with the highest doses used in previous studies (6, 11–13). Mice were monitored everyday for signs of disease, generally manifesting itself as anemia, loss of weight, and a hunched appearance.

Two experiments were performed: one where mice were killed at 150 days of age and one where mice were killed where they showed signs of disease.

Permanent Administration of Aspirin. Matings segregating for progeny which were *Apc*$^{Min/+}$, *Msh2*$^{-/-}$ or (*Apc*$^{Min/+}$, *Msh2*$^{-/-}$) were placed on aspirin diets containing either 0 or 400 mg/kg of aspirin before conception and throughout pregnancy and lactation. Progeny were weaned onto appropriate diets. Mice were killed when they showed signs of disease.

Histological Analysis. Tissues were removed, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 10 um, and stained with H&E before microscopic analysis. Scoring of intestinal lesion was achieved by removing the entire intestine at necropsy, flushing with PBS, and mounting *en face*. These preparations were then fixed in methacarn (4:2:1, methanol: chloroform:glacial acetic acid). Lesions were then scored macroscopically. Intestine was then wound into a "swiss" roll, which was subsequently embedded in paraffin and then sectioned as above.

Mutation Frequency at the *Dlb-1b* Locus. The *Dlb-1* assay was performed as described previously (16). For this assay, experimental cohorts were derived by backcrossing the *Msh2* mutants to two different C57Bl/6 strains, one of which was homozygous for the *Dlb-1 a* allele and one of which was homozygous for the *Dlb-1 b* allele. Mice were subsequently intercrossed from

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[3] The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; FAP, familial adenomatous polyposis; Cox2, cyclooxygenase 2; Dlb-1b, dolichos biflorus.

these two lines to generate mice heterozygous at the *Dlb-1* locus and segregating for all possible *Msh2* genotypes.

Results and Discussion

Aspirin Does Not Suppress Intestinal Neoplasia when Administered at Weaning. Wild-type and Apc^{Min} mice were placed on aspirin-containing diets (either 200 mg/kg or 400 mg/kg) at weaning. (Fig. 1). Mice exposed to the aspirin diet gained weight normally when compared with mice on the control diet, and no ulceration or intestinal pathology such as perforation of the intestine was observed in any of the wild-type mice treated with aspirin. Two independent analyses were performed.

First, cohorts of mice either wild-type or heterozygous for the Apc^{Min} allele were killed at 150 days of age, and the adenoma burden was assessed on whole mount preparations of the entire small intestine. No adenomas were seen in wild-type mice. Adenoma burden was as follows: for mock treated Apc^{Min} mice (n = 5), 9.8 (±/6.3); for mice treated with 200 mg/kg (n = 7), 9.7 (±5.88); and for mice treated with 400 mg/kg of aspirin (n = 5), 8.2 (±6.05). There was no difference in tumor burden at either 200 mg/kg (P = 1.00) or at 400 mg/kg (P = 0.75; Mann Whitney) compared with mock-treated mice, indicating that aspirin exposure was not modifying the Apc^{Min} phenotype.

Second, cohorts of mice were permitted to age until they developed obvious symptoms of intestinal neoplasia, usually bleeding from the anus or anemia scored through whitening of the feet. Fig. 1*A* shows a Kaplan-Meier plot reflecting survival over a 400-day period. Exposure to aspirin at either 200 mg/kg or 400 mg/kg did not alter the survival profile, again indicating that exposure to aspirin did not modify the Apc^{Min} phenotype.

Aspirin Suppresses Intestinal Neoplasia when Administered *in Utero*. Because Shoemaker *et al.* (14) and Reitmair *et al.* (15) have argued that the majority of adenomas are fixed before 6 days of age, we investigated the effect of aspirin exposure throughout embryogenesis and weaning. Cohorts of wild-type and Apc^{Min} heterozygotes were derived from parents placed on aspirin-containing diets before conception. Dietary exposure to aspirin was maintained throughout and beyond weaning. We first wished to establish whether *in utero* exposure resulted in increased embryonic lethality of Apc^{Min} heterozygotes, as has been reported for the Cox1- and Cox2-inhibitor piroxicam (17). Analysis of offspring showed this not to be the case, because there was no reduction in the number of Apc^{Min} heterozygotes in progeny from aspirin-exposed parents (P = 0.2; χ^2 test).

We next determined the survival profiles of each cohort (Fig. 1*A*), which showed a significant increase in survival in Apc^{Min} heterozygotes exposed to aspirin from conception onwards (P = 0.0004; log-rank test). This effect was sufficient to completely prevent the development of symptoms associated with intestinal neoplasia in 5 of 16 Apc^{Min} at 500 days of age. Analysis of tumor burden and distribution in those mice that developed intestinal tumors showed no obvious differences in either small (Fig. 1*B*) or large intestine (data not shown).

A number of different possibilities may underlie the discrepancies between the published studies examining the effect of aspirin on Apc^{Min}-associated tumorigenesis. First, that it is a consequence of differences in the level of aspirin exposure—a possibility which seems unlikely given the similar levels used in each experiment. Second, that it is attributable to differences in the genetic background of the mice used, either at known modifiers of the Apc^{Min} phenotype, such as *Mom-1*, or at as-yet unidentified modifiers (18). In this respect, it is notable that our study differs from previous studies by using mice homozygous for the relatively resistant C57/Bl6 *Mom-1* allele. Third, that it is a consequence of different husbandry regimes; for example, mice housed in a sterile facility may respond very differently to those in a nonsterile facility.

Comparison of the data presented here with published experiments does not permit us to distinguish between these possibilities, but it does serve to underline the relatively fragile nature of aspirin-mediated suppression within this model. We show here that one of the key modifiers of the effectiveness of aspirin is the time point of exposure. By increasing the period of dietary exposure to include *in utero* and perinatal exposure, we have dramatically enhanced the ability of aspirin to modify the Apc^{Min} phenotype. Within our experimental design, this enhancement is particularly notable given the failure to observe any effect in the Apc^{Min} cohort treated postweaning.

These findings are consistent with the presence of a developmental "window" for adenoma formation either *in utero* or shortly after birth. In support of this, Shoemaker *et al.* (14) showed that perinatal exposure to chemical carcinogens specifically enhances intestinal tumor development in the adult. One possible explanation for this phenomenon is the huge expansion in the number of intestinal crypts which occurs 3 weeks after birth. This process of crypt fission inevitably

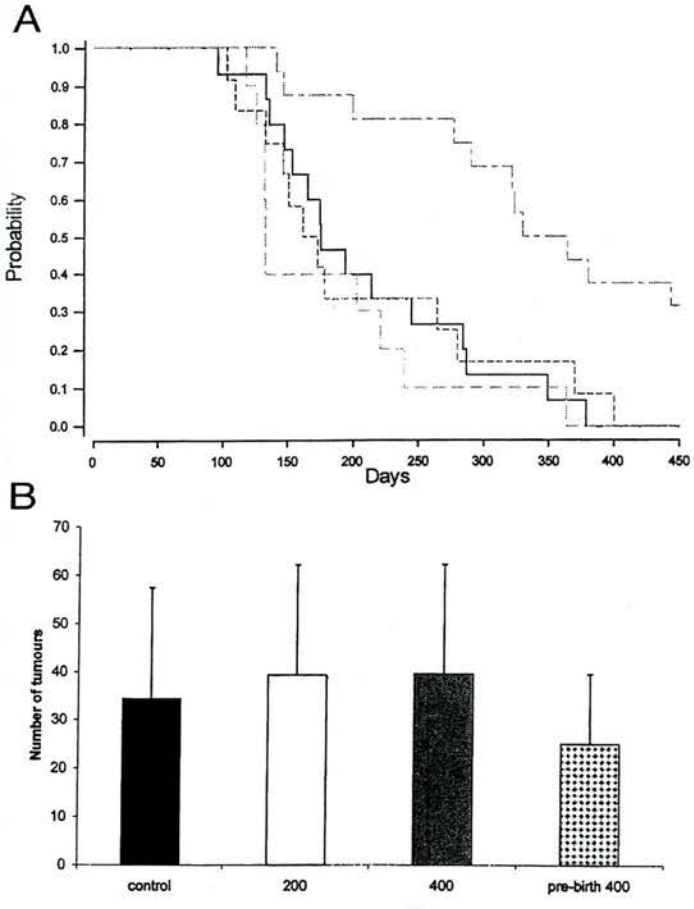

Fig. 1. *A*, Kaplan-Meier plot of survival of $Apc^{Min/+}$ exposed to control or aspirin-containing diet. *Black line*, mock treated mice (n = 15); *red line*, 200 mg/kg diet (n = 12); *blue line*, 400 mg/kg of diet (n = 10). There was no significant difference in the survival curves of either the mice treated with 200 mg/kg (log-rank test; P = 0.96) and 400 mg/kg (log-rank test; P = 0.30). *Green line*, $Apc^{Min/+}$ mice exposed to aspirin-containing diet from point of conception. These mice (n = 16) showed a significant increase in survival (log-rank; P = 0.0004) compared with mock-treated and aspirin-treated mice at weaning. *B*, tumor burden of mice at point of sacrifice as determined by onset of overt symptoms of intestinal tumorigenesis. ■, mock-treated mice; □, mice treated with 200v mg/kg; ▨, mice treated with 400 mg/kg of aspirin at weaning; ▦, mice treated with 400 mg/kg of aspirin from point of conception. There was no significant difference in the number of tumors at sacrifice between mock-exposed mice (n = 15) and mice exposed to 400 mg/kg from conception (Mann-Whitney; P = 0.613; n = 9). Tumor burden of mice >500 days of age were excluded from this analysis, because these mice were killed when symptomatic of lymphoma and not intestinal neoplasia.

expands the number of any mutation-bearing crypts present within the intestine and may therefore clonally expand any crypts bearing mutations at the *Apc* locus.

The presence of a very early window for neoplastic development raises serious questions about our understanding of *Apc*-mediated tumorigenesis. Loss of Apc function is considered to be the key initiating event in intestinal neoplasia and is strongly associated with both dysplasia and dysregulation of β-catenin [*e.g.*, Kongkanuntn *et al.* (19)]. However these phenomena simply are not observed in mice aged <4 weeks, indicating either that loss of *Apc* alone is insufficient to lead to the up-regulation of β-catenin and development of dysplasia, or that loss of *Apc* (and thereby dysregulation of β-catenin) occurs as a secondary event. In this regard we have shown previously that a proportion of dysplasias occurring in a mismatch repair-deficient background are not characterized by increased β-catenin levels (19).

Aspirin clearly acts to suppress these early events either by deleting or suppressing the phenotype of those cells which carry an increased predisposition to adenoma formation. This seems unlikely to be through modulation of β-catenin levels, as has been suggested by Mahmood *et al.* (6), because, as stated above, elevated levels of β-catenin are not observed in young animals. A second possibility is that aspirin may suppress neoplasia through a Cox2-mediated pathway. Cox2 up-regulation is observed in 80–85% of human colorectal carcinomas, in 50% of colorectal adenomas, and within tumors arising in the Apc^{Min} model (3). However, it seems unlikely that the primary effect of *in utero* aspirin exposure is mediated through Cox2 suppression, inasmuch as Cox2 overexpression is associated with the later stages of tumorigenesis by modulating angiogenesis and levels of apoptosis within tumors (3).

Whatever the mechanism of action of aspirin, these studies demonstrate that the initiation of adenoma formation occurs very early in the Apc^{Min} mouse, and that aspirin exposure at this time point can efficiently suppresses this process. Understanding the factors determining the predisposition to adenoma formation during embryonic development and perinatally might lead to greater insights into the molecular mechanisms of cancer in humans.

Aspirin Weakly Suppresses Neoplasia though not Mutation in $Msh2^{-/-}$ Mice. Having established an effective protocol for aspirin exposure in the murine model of FAP, we determined whether this approach could modulate the phenotype of the murine model of hereditary nonpolyposis colorectal cancer. A significant subset of human intestinal tumors are characterized by mutations in the MMR pathway, and all murine models of MMR deficiency show increased predisposition to neoplasia.

Cohorts of $Msh2^{-/-}$ mice were either exposed to aspirin or were fed control diet from conception, and Kaplan-Meier survival curves were generated (Fig. 2*A*). All 18 animals on the control diet were killed after the development of lymphoma, although 2 animals also had coexistent intestinal malignancy. All $Msh2^{-/-}$ mice exposed to aspirin also developed lymphoma; however, there was a small increase in survival compared with controls ($P = 0.05$; log-rank test). This slight shift in survival may reflect weak suppression of either intestinal malignancy or lymphomagenesis. This latter possibility is consistent with the one report that showed that aspirin exposure can result in the deletion of MSI-unstable cells in culture (9).

These results prompted us to determine directly whether aspirin exposure can suppress *in vivo* mutation in a mismatch repair-deficient background. Mutation frequency was scored at the *Dlb-1b* locus in *Msh2* mutant mice continually exposed to either control or aspirin-containing diets. Using this assay, we have shown previously that *Msh2*-deficient mice have a mutator phenotype at the *Dlb-1b* locus (16, 20). *Msh2*-deficient mice were analyzed at 4 months of age after exposure to either control or aspirin-containing diet. No difference in mutation frequency was observed at this time point, demonstrating that this regime of aspirin exposure does not modify the mutator phenotype of *Msh2*-null epithelium (Fig. 2*B*).

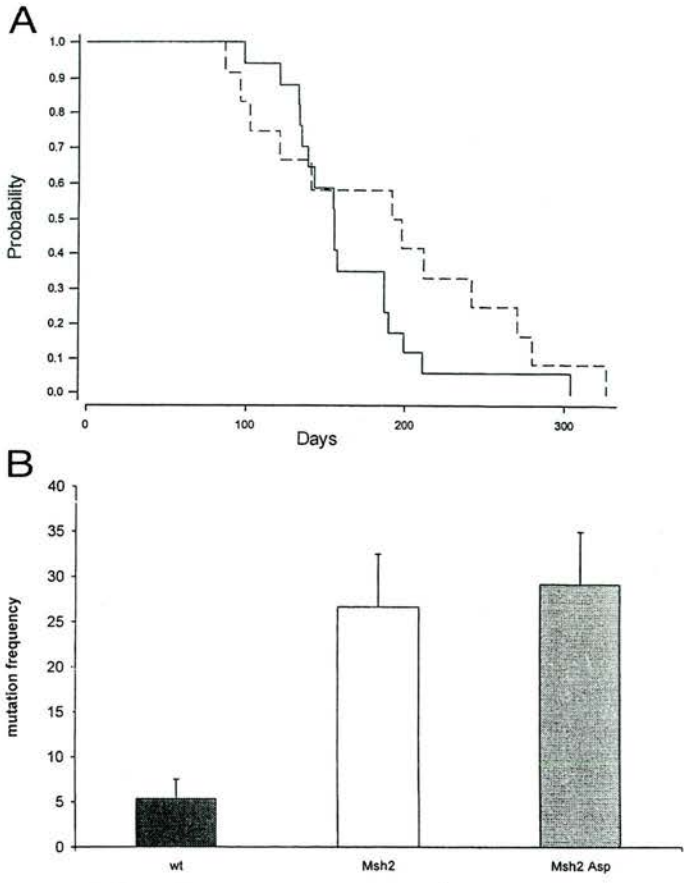

Fig. 2. *A*, Kaplan-Meier plot of survival of $Msh2^{-/-}$ mice exposed to control or aspirin-containing diet from point of conception. ———, mock-treated $Msh2^{-/-}$ mice ($n = 18$); ----, 400 mg/kg-treated $Msh2^{-/-}$ mice ($n = 20$). $Msh2^{-/-}$ mice treated with aspirin showed a weak increase in survival (log-rank test; $P = 0.05$). *B*, *in vivo* mutation frequency/10,000 villi at the *Dlb-1b* locus after aspirin treatment. Mutation frequencies were determined from intestinal wholemounts at 4 months of age. *Columns*, mean mutation frequency at the *Dlb-1b* locus. At least three mice were used per each column. *Bars*, SD. ■, mock-treated wild-type mice; □, mock-treated $Msh2^{-/-}$ mice; ▨, $Msh2^{-/-}$ mice exposed to 400 mg/kg of dietary aspirin from point of conception. There was not a significant reduction in mutation frequency after aspirin treatment ($P = 0.77$; Mann-Whitney).

Aspirin Suppresses Intestinal and Mammary Neoplasia in $Apc^{Min/+}$ $Msh2^{-/-}$ Mice. In human colorectal cancer MMR-deficient tumors differ in a number of respects to MMR-proficient tumors. For example, MMR-deficient tumors express lower levels of Cox2 as well as exhibiting very different patterns of mutation and genomic instability. These differences invoke different mechanisms for MMR-driven neoplasia, raising the possibility that aspirin exposure may have an additional effect in a MMR-deficient background, for example through the deletion of cells showing MSI.

The development of lymphoma in $Msh2^{-/-}$ mice largely precludes an analysis of the effect of aspirin upon *Msh2*-dependent intestinal neoplasia (22). However, *Msh2* deficiency has been shown to greatly accelerate intestinal neoplasia in the Apc^{Min} mouse (15), so permitting an analysis of the effect of aspirin in this context. In addition, mammary akanthoma occur in $Apc^{Min/+}$ mice (23), and this is greatly accelerated in ($Apc^{Min/+}$, $Msh2^{-/-}$) mice, permitting a study of the effect of aspirin on the development of this lesion (see below). Cohorts of ($Apc^{Min/+}$, $Msh2^{-/-}$) mice were generated and exposed to either control diet or aspirin-containing diet from conception onwards, and Kaplan-Meier survival curves were generated (Fig. 3*A*). Survival was markedly enhanced after aspirin expo-

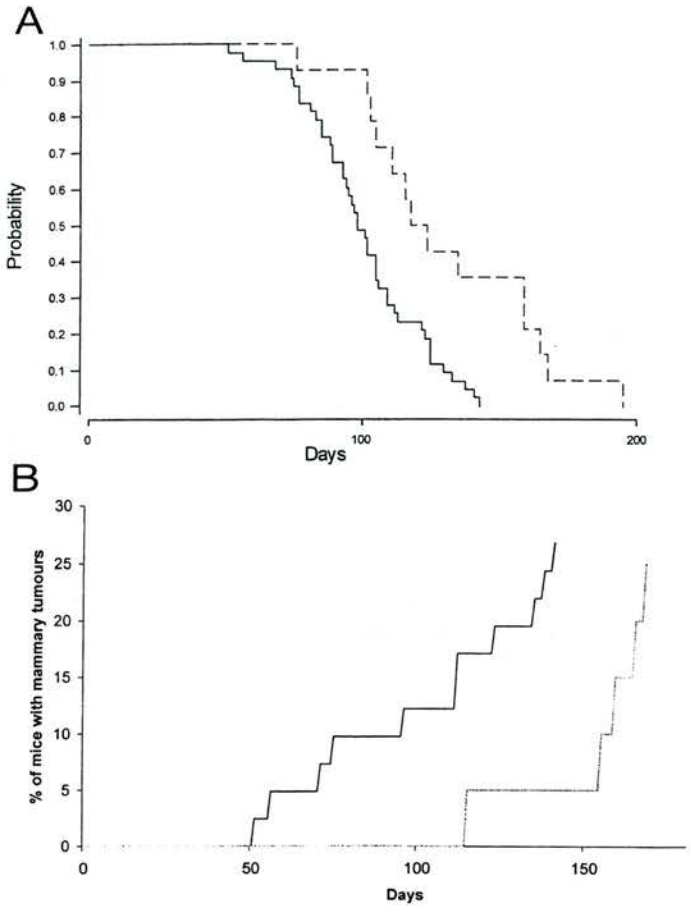

Fig. 3. *A*, Kaplan-Meier plot of survival of $Apc^{Min/+}$ $Msh2^{-/-}$ mice exposed to control or aspirin-containing diet from point of conception. ———, mock-treated mice (n = 43); ----, mice treated with 400 mg/kg from conception (n = 17). Aspirin treatment significantly increased survival (log-rank test; P = 0.0001). *B*, graph showing delayed incidence of mammary tumorigenesis in the Min Msh2 mice. ———, mock-treated mice; , mice treated with 400 mg/kg of aspirin from conception.

sure (log-rank test; P = 0.0001), although it was still reduced by comparison to untreated Apc^{Min} heterozygotes. Aspirin exposure did not alter either tumor distribution or burden scored at the point of death (data not shown).

In the cohorts maintained on the control diet, a single mammary akanthoma was observed in a total of 15 $Apc^{Min/+}$ mice, whereas none were detected in 18 $Msh2^{-/-}$ mice. Development of this tumor type was enhanced in $Apc^{Min/+}$ $Msh2^{-/-}$ mice (11 tumors in 41 mice). In the cohorts exposed to aspirin, no mammary akanthomas were observed in 10 $Apc^{Min/+}$ mice, whereas a single akanthoma was observed in 21 $Msh2^{-/-}$ mice. In the ($Apc^{Min/+}$, $Msh2^{-/-}$) cohort, five mammary akanthomas were observed in 20 mice. All of these mice also had intestinal neoplasia. Although this represents a similar prevalence to that observed in the untreated ($Apc^{Min/+}$, $Msh2^{-/-}$) cohort, the onset of akanthoma was significantly retarded in the aspirin-treated mice (Fig. 3*B*). These results therefore show aspirin exposure significantly delays the onset of mammary tumorigenesis in the ($Apc^{Min/+}$, $Msh2^{-/-}$) background, consistent with several recent studies which have shown that both mutation of *Apc* and overexpression of Cox2 can lead to mammary neoplasia (24), and, furthermore, that selective Cox2 inhibitors can suppress rodent mammary tumorigenesis (25).

Overall, the median life span of mock-exposed Apc^{Min} mice was 175 days compared with 330 days for aspirin-treated mice, an increase of 89% or 155 days. The comparable figures for the ($Apc^{Min/+}$, $Msh2^{-/-}$) cohort are 98 days (mock-treated) and 127 days (exposed), an increase of 29% or 29 days. This comparison suggests that the effect of aspirin was no greater in the ($Apc^{Min/+}$, $Msh2^{-/-}$)background. Taken together with the weak effect of aspirin on survival of $Msh2^{-/-}$ mice and the failure to observe an influence of aspirin upon mutation rate, these data argue that aspirin specifically suppresses tumorigenesis in an *Apc*-dependent manner. This conclusion contrasts the observation that *in vitro* aspirin can delete cells characterized by MSI (9). This contradiction may be explained by the surprising fact that adenomas developing in ($Apc^{Min/+}Msh2^{-/-}$) mice do not show microsatellite instability (15), which was independently confirmed here (data not shown). This demonstrates that gross microsatellite instability is not required to drive adenoma formation in this background, possibly because of the high selective pressure for adenoma formation consequent upon the Apc^{Min} mutation. Thus, somewhat paradoxically, cells within the ($Apc^{Min/+}Msh2^{-/-}$) mice do not exhibit gross levels of MSI and therefore are not available as targets for aspirin-mediated deletion.

In summary, we have shown that dietary aspirin exposure can suppress tumorigenesis in the murine intestine and the mammary gland. This effect seems to be specifically associated with a loss of function of Apc and seems to only weakly modify the MMR phenotype. Critically, in the intestine, suppression only becomes apparent if exposure covers the period between conception and weaning. Obviously prophylactic treatment with aspirin of FAP patients during this window would be inappropriate because of the well-known deleterious side effects of aspirin. This, therefore, identifies a potential window of opportunity for the chemoprevention of intestinal neoplasia. The challenge will now be to establish whether NSAIDS (other than aspirin) can be therapeutically effective within this window.

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Msh-2 suppresses *in vivo* mutation in a gene dose and lesion dependent manner

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Mice deficient for the mismatch repair (MMR) gene *Msh2* show accelerated tumourigenesis and a reduced apoptotic response to DNA damage of methylation type. Here we examine the effect of mutation for *Msh2* on *in vivo* mutation frequencies in the intestine as determined by loss of function at the *Dolichos biflorus* (*Dlb-1*) locus. Spontaneous mutation frequencies were scored in cohorts of ageing mice either wild type or mutant for *Msh2*. In mice less than 1 year old, mutation frequencies were only elevated in *Msh2* null mice. However, beyond this age heterozygous *Msh2* mice showed significantly higher mutation frequencies than controls. These findings implicate a gene dose dependent requirement for *Msh2* in mutation suppression and prompted an analysis of young *Msh2* mutants following exposure to DNA damage. Following exposure to N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), *Msh2* deficient mice show a reduced apoptotic response and an increase in mutation frequency. Heterozygotes did not differ from controls. Following exposure to cisplatin, no significant elevation was seen in mutation frequencies, even within homozygotes. This is particularly surprising given the association between cisplatin resistance and MMR deficiency. These findings therefore demonstrate a complex reliance upon functional *Msh2* in mutation surveillance. We have identified three separate scenarios. First, where retention of both *Msh2* alleles over an extended period of time appears critical to the suppression of spontaneous mutation; second, 3 weeks following exposure to MNNG, where only complete loss of *Msh2* results in elevated mutation; and finally following cisplatin exposure, where induced levels of mutation are independent of *Msh2* status. *Oncogene* (2001) **20,** 3580–3584.

Keywords: mismatch repair; apoptosis; mutation; heterozygosity; cisplatin; alkylation

Structural distortions produced by nucleotides which are either unpaired or paired with non-complementary nucleotides are recognized by proteins encoded by the mismatch repair genes. Several members of this family of genes have been characterized within *Saccharomyces cerevisiae*, which led to the identification of mammalian homologues. Six human mismatch repair genes have been cloned, MSH2, MLH1, PMS1, PMS2, MSH3 and MSH6 (Kinzler and Vogelstein, 1996; Wheeler *et al.*, 2000). Constitutive inactivation of these genes has been associated with the development of cancer, the best characterized relationship being between the inherited cancer-susceptibility syndrome of Hereditary Nonpolyposis Colorectal Cancer (HNPCC) and germline mutations in MSH2, MLH1 and PMS2 (Nicolades *et al.*, 1994; Kinzler and Vogelstein, 1996). Mice have been produced bearing targeted inactivations of the *Mlh1*, *Msh2*, and *Pms2* genes. Homozygous mice of these mutant strains are viable but prone to the development of different types of neoplasia. All of these mutant strains develop lymphoma, however they differ in their susceptibility to intestinal neoplasia, which only develops in *Mlh1* and *Msh2* deficient mice (De Wind *et al.*, 1995; Reitmair *et al.*, 1996; Prolla *et al.*, 1998). As such, *Msh2* and *Mlh1* homozygotes can be considered reasonably good models of HNPCC. By contrast, mice heterozygous for *Msh2* and *Mlh1* do not show reduced survival compared to wild type controls, nor are they predisposed increased intestinal neoplasia. They are, however characterized by increased tumorigenesis. Surprisingly when these tumours were examined for Loss of Heterozygosity (LOH) only one out of 71 had lost the remaining copy of *Msh2* and this was the only tumour to exhibit microsatellite instability (MI), indicating that the majority of tumours retained heterozygosity for *Msh2* (De Wind *et al.*, 1998). These results are therefore consistent with a heterozygous effect of Msh2.

There are currently two possible biological mechanisms underlying the relationship between MMR deficiency and neoplasia. The first of these arises out of the MMR proteins defining role in mediating DNA repair. MSH2 recognizes mismatched nucleotides as a heterodimer with MSH6 and insertion/deletion loops with MSH3 (Buermeyer *et al.*, 1999). In what is assumed to be a direct consequence of failure of these processes, *Msh2*−/− cells exhibit a mutator phenotype and microsatellite instability (De Wind *et al.*, 1995; Reitmair *et al.*, 1997). These data support the concept

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that failed recognition and repair of mismatch lesions leads directly to an increase in mutation frequency and thereby to malignancy. A second possible mechanism derives from recent studies which have shown that loss of the mismatch repair gene *Msh2* impairs the apoptotic response to alkylating agents in a variety of systems (Toft *et al*., 1999; Hickman and Samson, 1999). Consistent with this, cells deficient in MMR have been shown to be resistant to a number of clinically important drugs, such as cisplatin, temozolomide and doxorubicin (Fink *et al*., 1998). This raises the possibility that increased mutation frequencies may partly arise from failed clearance of DNA damage bearing cells. Currently, there is little direct evidence in support of this hypothesis, although it is clear that *Msh2* deficiency does lead to an elevation in mutation frequencies following exposure to DNA damage (Andrew *et al*., 1998; Toft *et al*., 1999). Toft *et al*. (1999) have demonstrated that (in a model system) a component of this increase is attributable to increased cell survival. Thus, although Msh2 clearly plays a role in mediating cell death, the precise relationship between the ability to engage apoptosis and mutation surveillance remains relatively poorly defined.

To investigate the *in vivo* consequences of heterozygosity at the *Msh2* locus and to further explore the relationship between apoptosis and mutation surveillance, we have examined the gene dependency of these two endpoints in the small intestine of mice wild type, heterozygous and nullizygous for *Msh2*. These studies were performed both at spontaneous levels of damage and following exposure to the DNA damaging agents MNNG and cisplatin.

We first scored mutation frequency at the *Dlb-1* locus at spontaneous levels of DNA damage over a 13 month time course (Figure 1). The *Dlb-1* assay was performed as previously described (Winton *et al*., 1988). For this assay, experimental cohorts were derived by backcrossing the *Msh2* mutants to two different C57Bl/6 strains, one of which was homozygous for the *Dlb-1a* allele and one of which was homozygous for the *Dlb-1b* allele. Mice were subsequently intercrossed from these two lines to generate mice heterozygous at the *Dlb-1* locus and segregating for all possible *Msh2* genotypes. Loss of the *Dlb-1b* allele was then scored per 10 000 villi.

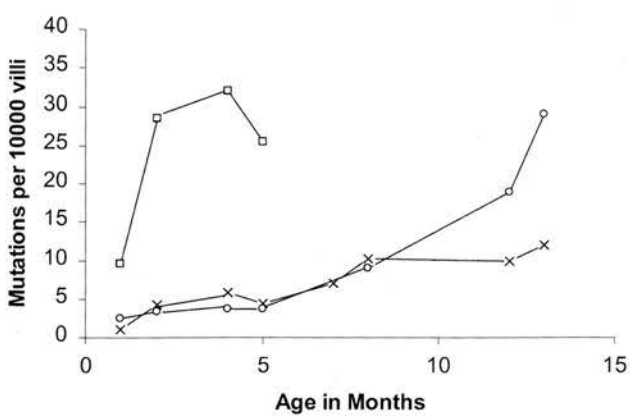

Figure 1 Spontaneous Dlb-1 mutation frequencies scored per 10 000 villi in mice wild type (crosses), heterozygous (circles) and homozygous null (square points) for *Msh2*. Each point represents at least three mice. At ages over 1 year, heterozygous mice ($n=12$, mean = 24) have a significantly higher mutation frequency than wild type ($n=8$, mean = 11) mice. $P=0.04$, Mann Whitney U test)

Msh2 null mice exhibited a higher mutation frequency than wild type controls and *Msh2* heterozygous mice. *Msh2* null mice showed a marked increase in mutation frequency from 4 to 8 weeks: a period where there is a significant increase in the number of crypts (Shoemaker *et al*., 1995). Surprisingly, beyond this time point there was no further increase in mutation frequency. This may reflect selection for mice with a reduced predisposition to neoplasia (and potentially reduced mutation frequencies) at the later time points as the peak incidence of neoplasia occurs at 3–4 months.

The most intriguing result is the increase in mutation frequency in the heterozygotes aged over 12 months. Similar results have been shown using very sensitive mutation screens in *Msh2* and *Mlh1* heterozygous diploid yeast (Shcherbakova and Kunkel, 1999; Drotschmann *et al*., 1999, 2000). The authors of these studies argued that this increased mutation frequency occurred due to loss of heterozygosity in a small population of the heterozygote cells. However, although LOH at the *Msh2* locus cannot be formally ruled out as the mechanism underlying the increase in the aged hemizygotes, we believe this to be unlikely as this requires two mutations (inactivation of the remaining *Msh2* allele and subsequent inactivation of *Dlb-1b*) to occur sequentially within the same stem cell. Wild type cells accumulate on average one additional mutation per 10 000 crypts each month (Winton *et al*., 1988). If we assume a null hypothesis and that heterozygosity for *Msh2* plays no direct role in suppressing mutation, and furthermore that *Msh2* is inactivated at a similar rate to *Dlb1*, then after 13 months there would be approximately 13 *Msh2* null clones per 10 000 crypts. Our analysis shows *Msh2* deficiency increases the mutation frequency to, at maximum, less than 10 times that found in wild type cells (the highest fold difference of 9.6 was observed at 1 month). This indicates that the additional mutation burden from the *Msh2* null clones would be less than 1 mutation per 10 000 villi. This increase is clearly insufficient to account for the actual observed increase, strongly implying that the elevation in mutation frequency is occurring as a direct consequence of *Msh2* heterozygosity.

We interpret the effect of heterozygosity at the *Msh2* locus as leading to a constant but small increase in mutability which only becomes evident over a long period of elapsed time (in this instance 12 months of age). However, it remains formally possible that heterozygosity for *Msh2* may result in the mice becoming hypermutable at old age, a hypothesis which we have not addressed in this study, but one which could be addressed by future experiments.

The data presented here parallels other studies which have suggested gene dose sensitivity for Msh2 dependent responses. These include analyses of the level of DNA lesions and the apoptotic response following exposure to low level radiation treatment (DeWeese *et al*., 1998), and also by studies which have shown very low rates of LOH at the Msh2 locus in tumours arising in Msh2 hemizygotes (De Wind *et al*., 1998).

The above observations prompted analysis of the apoptotic response, to determine if this response was also *Msh2* gene dose sensitive. Apoptosis of enterocytes within the crypts of Lieberkuhn was examined at 6 h after drug exposure (Figure 2). This analysis was performed on mice aged 8–12 weeks. We have previously established that this time point coincides with the peak induction of apoptosis within this structure (see Toft *et al*., 1999; Sansom and Clarke, 2000). Consistent with previous results *Msh2* deficient mice showed a reduced apoptotic response following exposure to MNNG whilst there was normal apoptotic response in wild type and *Msh2* heterozygous mice ($P=0.38$). Following exposure to cisplatin, no difference was observed between hemizygotes and wild type mice ($P=0.20$, Mann Whitney U), a result which is perhaps not unexpected as *Msh2* null mice show only a marginal reduction in the apoptotic response compared to wild type controls (Toft *et al*., 1999). The failure to observe high levels of MMR-dependent cell death following cisplatin exposure contradicts the scenario reported for ovarian cancer cells, where resistance to cisplatin is associated with loss of MMR function and restoration of MMR causes re-sensitization. This must either be a reflection of the rather subtle changes in the ability to engage apoptosis, or must implicate non-apoptotic MMR-dependent mechanisms in long term cell survival.

Despite the presence of a normal, intact apoptotic response in *Msh2* heterozygotes it remained possible that there could be a gene dosage effect in mutation induction following these agents. Previously Shcherbakova and Kunkel (1999) have demonstrated significant increases in mutation frequency in yeast heterozygous for *Mlh1* following exposure to either bleomycin or UV irradiation. These phenomena were assumed to arise from damage enhanced LOH. Furthermore, studies

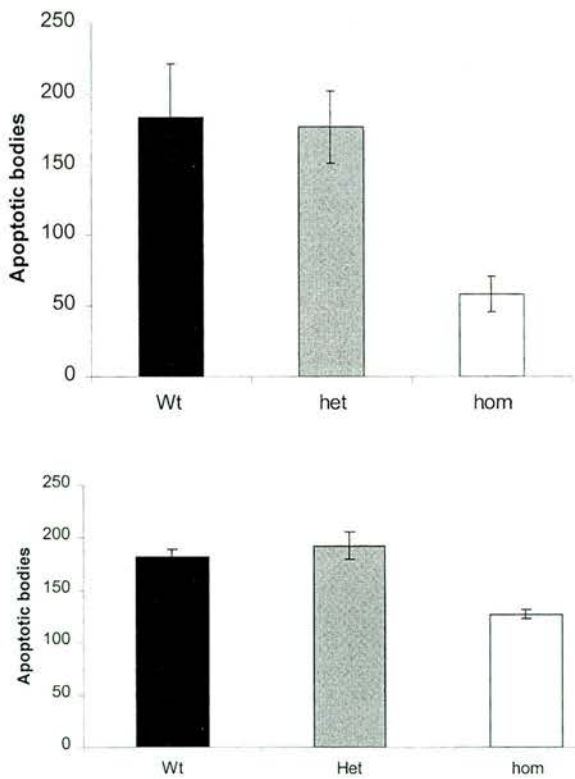

Figure 2 The *in vivo* apoptotic response at 6 h following drug exposure. For each animal 50 half crypts were analysed; the total number of apoptotic bodies scored in all crypts analysed is plotted on the vertical axis. (**a**) Following 50 mg/kg of MNNG, (**b**) following 10 mg/kg of cisplatin. Closed bars, wild type mice; grey bars, heterozygous mice and open bars, *Msh2* null mice. Columns represent mean, errors bar represents s.d. At least three mice were used for each point

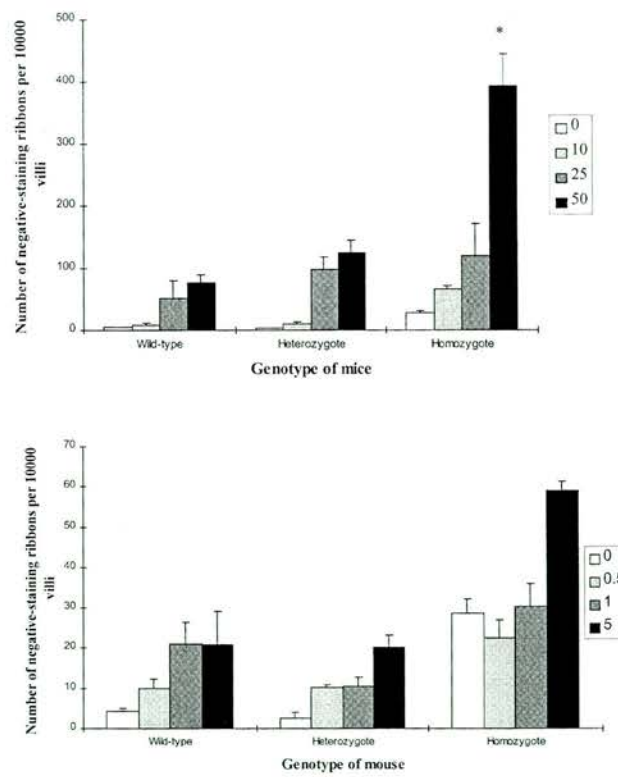

Figure 3 Induced *in vivo* mutation frequency at the *Dlb-1b* locus. Mutation frequencies were determined from intestinal wholemounts 21 days after ip drug injection: Columns represent mean mutation frequency at the *Dlb-1b* locus at the drug dosage shown. At least three mice were used per each column. Error bars represent s.d. (**a**) Mutation frequency following MNNG. Open bars, mock treatment; light grey bars, 10 mg/kg; dark grey bars, 25 mg/kg; closed bars, 50 mg/kg. At 50 mg/kg Msh2 nulls have a significantly higher mutation frequency than wild type and heterozygous mice ($P=0.04$, Mann Whitney U test). (**b**) Mutation frequency following cisplatin. Open bars, mock treatment, light grey bars, 0.5 mg/kg; dark grey bars, 1 mg/kg; closed bars, 5 mg/kg

using *Mlh1* heterozygous mice following MNU (N-methyl-N-nitrosourea) exposure have revealed increased rates of tumourigenesis compared to wild type controls (Kawate *et al*., 2000). We therefore investigated mutation frequency at the *Dlb-1* locus in young (8–12 weeks old) *Msh2* mutant mice following treatment with either MNNG (Sigma) or cisplatin (David Bull Laboratories) (Figure 3). Mutation was scored 3 weeks post i.p. injection. Following exposure to 50 mg/kg MNNG we observed a significant increase in mutation frequency in *Msh2* null mice compared to controls (Mann Whitney U test, $P=0.0414$), but not in heterozygotes (Mann Whitney U test, $P=0.17$). These results parallel those obtained following exposure to the alkylating agent Temozolomide (Toft *et al*., 1999), confirming that Msh2 normally suppresses mutation following damage of alkylation type but also showing that this response is not *Msh2* gene-dose sensitive. Following cisplatin exposure *Msh2* nulls showed increased mutation frequencies relative to controls and heterozygous mice, however these increases could entirely be accounted for by the increase in spontaneous mutation frequency. These results therefore fail to demonstrate a role for *Msh2* in mutation surveillance following cisplatin treatment and question the significance of mismatch repair in the clearance of cisplatin-induced DNA damage in normal cells. Our findings complement recent studies by Branch *et al*. (2000), who demonstrated that loss of MMR was only a minor contributor to cisplatin resistance in ovarian tumour cell lines.

The studies reported here have been performed in normal intestinal enterocytes. It remains possible that the reliance upon functional MMR for mutation surveillance markedly differs between normal and neoplastic cells. Pertinently, Strathdee *et al*. (2001) have shown that mouse embryonic fibroblasts deficient for *Msh2* still undergo a G2 arrest following cisplatin treatment in marked contrast to MMR deficient tumour cell lines which lose this checkpoint (Brown *et al*., 1997).

In conclusion, we demonstrate an extremely complex reliance upon *Msh2* in mutation surveillance. We show that *Msh2* is critical in monitoring spontaneous levels of DNA damage, such that even a 50% reduction in *Msh2* gene dosage can elevate mutation frequency. We show that this reliance upon *Msh2* in response to spontaneous levels of DNA damage is not directly reflected by gene dependencies in either the apoptotic response or mutation burden following acute DNA damage. We show that *Msh2* deficiency leads to an increase in the *in vivo* mutation frequency following MNNG treatment, but remarkably that this is not the case following exposure to cisplatin. Taken in the context of the recent proliferation of data relating to MMR-dependent DNA damage responses, these observations show that reliance upon functional MMR is highly lesion type dependent. They also challenge our understanding of MMR-dependent suppression of mutation, perhaps partly because MMR dependent responses are usually interpreted in an experimental setting following high, non physiological levels of DNA damage. Clearly, although such scenarios may directly relate to the response to chemotherapeutics in a clinical setting, they may not accurately reflect the reliance upon MMR in normal tissues exposed to low levels of spontaneous damage.

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Minireview

P53 null mice: damaging the hypothesis?

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Abstract

P53 is extremely well characterised as a tumour suppressor gene, and many activities have been attributed to it which are consistent with this function. However, despite being the subject of intense study it still remains unclear precisely which of these functions is crucial to its in vivo role as a tumour suppressor gene. This is particularly true of its role in the induction of apoptosis. The original observation of p53-dependent apoptosis gave rise to the following hypothesis: namely, that p53 deficiency leads to a persistence of DNA damaged cells which are the potential founders of malignancy. This review summarises the data for and against this hypothesis, with specific emphasis on data obtained from studies of the murine intestine. What emerges from these studies is a complex picture, where data can be obtained in support of this hypothesis, but there are many circumstances which exist where it is not supported. Taken together this collection of data suggests that the abrogation of p53-dependent apoptosis may indeed impact upon carcinogenesis and neoplastic progression, but that the simplistic notion of p53 as the single gatekeeper of this pathway is untenable.

Keywords: P53; Mice; Tumour suppressor gene

1. Introduction

Any search on p53 soon yields a staggering number of publications (in 1998 alone there were 3744 publications), highlighting that this is probably the most studied protein in scientific world. This statistic reflects the fact that p53 mutations are amongst the commonest molecular events in neoplasia: it is mutated in approximately 50% of human cancers and is possibly inactivated in all tumours through loss or upregulation of other genes in the p53 pathway, e.g. $p14^{ARF}$ and *Mdm2* [1,2].

For many years it has been known that p53 is a critical cellular mediator of the response to genotoxic damage in vivo and in vitro [3]. P53 has been shown to induce apoptosis (programmed cell death) in response to a wide variety of insults and shown in vitro to also induce G1 and G2 cell cycle arrest via transcriptional activation of p21/WAF [4].

Recently, a series of significant advances have been made in the in vitro characterisation of the p53 pathway. These include the identification of ever increasing types of cellular stress that upregulate p53; the identification of post-translational modifications in response to such stress, and the characterisation of proteins that interact with or are transactivated by p53 (see Fig. 1) (for a recent review see [5]). One obvious goal of these studies is to link stimuli, such as ionising

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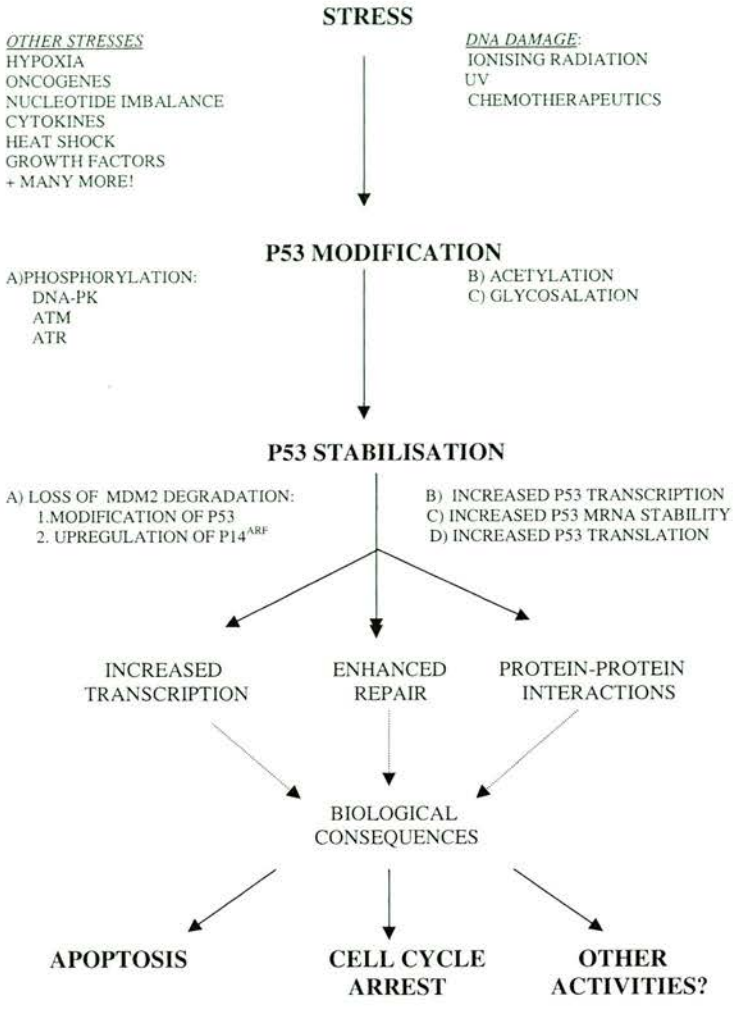

Fig. 1. Postulated p53 pathway, see [5]. Following a range of stresses, the p53 protein is modified. Thus far the proteins identified to modify p53 are mainly kinases: DNA-PK, ATM and ATR. Although as yet unproven, such modifications are postulated to render Mdm2 unable to bind p53, thus, causing accumulation of p53. Depending on the specific cellular context, this could lead to apoptosis, cell cycle arrest or even senescence (see [5] and references therein).

radiation to a particular set of events which determine biological fate, such as the engagement of apoptosis or cell cycle arrest. However, whether the system will be as straightforward as this remains to be seen. It has also been demonstrated in vitro that p53 may have a direct role in maintaining genome integrity by preventing homeologous recombination, recognising and removing mismatches and participating in Nucleotide Excision Repair [6,7].

As the number of different roles that p53 plays in vitro continues to proliferate, the question of in vivo relevance comes to the fore. For example, embryonic fibroblasts (MEFS) derived from p53 null mice do not senesce in vitro unlike their wild type (WT) counterparts, indicating that p53 plays an essential role in senescence. This implies that the ability to engage senescence may play a crucial role in tumour suppression. However, translating such a finding into a true in vivo setting can prove difficult. As yet, irrefutable evidence has not yet been provided that senescence plays a significant role in vivo. Indeed, an extreme viewpoint would be that senescence may not be an important in

vivo phenomenon, and even that it may be an artefact of the culturing procedure [8]. This represents an extreme interpretation of these difficulties, however, it serves to highlight the inherent difficulties in the study of a range of biological phenomena in vivo. Certainly the scope of in vivo analysis is currently restricted by the complexity of the in vivo setting and by the relatively limited availability of good in vivo tools.

This review will focus upon studies performed in vivo using p53 null mice and specifically on those experiments aimed at addressing the significance of p53 in inducing apoptosis following DNA damage. The observation that p53 deficiency abrogates the normal apoptotic response to DNA damage gave rise to the notion that the normal ability to engage apoptosis may underlie the role of p53 as a tumour suppressor. Hence, in the absence of functional p53 it was proposed that cells bearing DNA damage would persist, and that these 'undead' cells would carry an increased mutation burden, and therefore, be more likely to act as the founders of neoplastic clones [3]. Results addressing this hypothesis obtained from p53 null mice will be discussed and compared to similar data obtained from mice deficient in the mismatch repair gene *Msh2* (MutS homologue 2).

2. The phenotype of the p53-deficient mice

Three different strategies were employed to generate the first p53 knockout mice in the early 1990s [9–11]. All gave similar phenotypes with the majority of the p53 null mice dying from lymphoma at around 6 months. This gave definitive proof that p53 was a tumour suppressor gene, which previous clinical data and in vitro work had suggested [12].

Mice heterozygous for p53 can be viewed as a relatively accurate model of the Li-Fraumeni syndrome, as they develop tumours of a similar spectrum as those seen in Li-Fraumeni patients (oestosarcomas, lymphomas and soft tissue sarcomas) [13,14]. There are, however, two notable exceptions to this, namely, the high incidence of lymphoma in the mice and the absence of breast tumours (for recent reviews of mouse data see [15,16]). P53 heterozygotes develop symptoms of disease later than their p53 null counterparts, with approximately 50% of mice succumbing to tumours by 18 months. As wild type mice live for up to 36 months, this age of death of the heterozygotes is often compared to Li-Fraumeni patients where affected individual have a 50% chance of developing cancer by the age of 30 [17]. However, great care should be taken in making direct comparisons between the consequences of p53 deficiency in man and mouse as many species specific differences exist, perhaps most notably in the strong predisposition to lymphoma observed in the mouse.

Within the mouse, strain dependent differences in phenotypes have readily been observed [18,19]. For example, pure bred 129SV p53 null mice die from tumours much earlier than those crossed onto a C57Bl6 background. Furthermore, 50% of p53 null males in 129SV develop testicular teratomas which are only observed rarely in other backgrounds. This latter observation probably reflects that p53 deficiency accentuates any natural tumour predisposition, as wild type 129SV mice show a spontaneous predisposition to testicular teratomas compared to other strains [19–21].

3. P53 and development

Prior to the development of mice deficient for p53, a number of different in vitro studies had suggested key roles for p53 in initiating both apoptosis and cell cycle arrest. These predicted a severe phenotype for p53 deficiency, and it was therefore, of some surprise that p53 deficiency did not result in embryonic lethality as had been reported for other cell cycle genes, such as Retinoblastoma (*Rb*) [22]. This led to the suggestion that p53's true role was as a 'guardian of the genome', with a primary function of protecting against genotoxic damage [23]. Any role for p53 in development was therefore initially discounted, however, several lines of evidence have now made it clear that p53 does indeed play a role in normal embryonic development.

A common misconception when trying to understand the function of tumour suppressor genes is to presuppose that their sole function lies in the prevention of malignancy. However, if this were the case it is difficult to understand the common inheritance of tumour suppressor genes across many organisms that are clearly not prone to malignancy. Moreover, it is difficult to identify the selection pressure for retention of such a class of genes if the phenotype of cancer is only manifested post-reproduction [24]. These points

argue for other primary roles for tumour suppressor genes, presumably based around their function in suppressing mutation and genomic instability. This can be most easily rationalised in terms of a role in suppressing mutation within the germ line, as accumulation of mutations in the germ line would clearly compromise the organism. By comparison, the accumulation of mutations in the somatic cells (especially post reproduction) are unlikely to effect the fitness of the organism (Disposable Soma theory of ageing see [25]). Taken together, these considerations argue in favour of a general role for tumour suppressor genes either in the germline or during embryogenesis.

This argument may be applied to tumour suppressor genes other than p53, such as the mismatch repair genes *Msh2* or *Mlh1*. Loss of function of these genes leads predominantly to colorectal or ovarian cancer, however, the primary function of these genes is to maintain genome integrity by removing mismatches and insertion/deletion loops [26]. If this were to occur in the germ line, mutations would gradually accumulate leading to a decline in fitness (e.g. smaller litters due to lethal mutations). In this respect it will be interesting to determine if the phenotype of mice mutant for given tumour suppressor genes is modified as the number of generations increase.

These considerations suggest that if p53 is truly a guardian of the genome, it is likely to play an important role in the germ line and in the developing embryo. Such a role is supported by the reported strain dependent sub-fertility seen in the p53 mutant mice [27,28]. This led Kondo [24] to propose that the major role for p53 was in 'apoptotic repair' (the deletion of cells bearing DNA damage through apoptosis) principally within the germ line. Data has been obtained in support of a role in embryogenesis from the careful analysis of p53 breeding data. This indicated an apparent deficiency in the number of live born p53 null female mice [29,30]. When embryos were examined in utero and at the point of parturition, 23% of female null embryos were found to have neural tube defects, which presented as exencephaly from approximately day 12 of development, ultimately resulting in an encephaly at birth. Further support of the concept that p53 might suppress germline mutations comes from the observation that a significant increase (60%) in exencephaly is seen in p53 null female progeny derived from males irradiated prior to mating. Again this suggests that p53 plays an important role in protection of the embryo against genotoxic stress. The reason why this manifests itself predominantly within females remains unclear, but this does reflect a similar sex bias seen in some human neural tube defects.

The actual levels of p53-dependent exencephaly have been found to be dependent on genetic background. One apparently extreme example of this is the background dependent variation in lethality seen in mice null for both p53 and *Msh2* using a C57Bl6 background Cranston et al. [31], showed that no doubly mutant females survived to birth. We have performed similar experiments using an outbred strain, and although we did observe a relative deficiency of doubly null females this was no greater than the deficiency due to loss of p53 [32]. Recently, we have crossed the p53 null strain onto a C57Bl6J background and find that the levels of exencephaly in females increases dramatically, such that <5% of surviving p53 nulls are female. This level of defect has been sufficiently high to preclude any confirmation of the results obtained by Cranston et al. [31] within our own stocks.

Further support for a role for p53 in protecting the embryo against genotoxic stress comes from Normiura et al. [33], who demonstrated that if p53 +/+ fetuses were irradiated at day 9.5 with 2 Gy of irradiation, many would die in utero and that those born would be subject to a low frequency of malformation. However, similarly treated p53 −/− fetuses showed a higher proportion of surviving embryos, but these were subject to a greater number of deformities. This in itself does not prove Kondo's speculative claims but it does highlight that p53 is important in development after genotoxic insult.

These studies have demonstrated at least partial dependency upon p53 for normal development. Our understanding of this role has now been extended by experiments using mice null for Mdm2 which have shown that abnormal regulation of p53 is also incompatible with normal development [34,35]. On its own, Mdm2 deficiency results in lethality, yet this is rescued in the absence of p53. The basis of this rescue is assumed to lie in the ability of Mdm2 to target p53 for degradation. Thus, in the absence of functional Mdm2, p53 is overexpressed resulting in lethality (see Fig. 1). Similar evidence for interaction between these two genes has been obtained using a transgenic

approach to overexpress a Mdm2 transgene and so cause loss of p53 [36].

4. Measuring mutation frequency in vivo

The fact that the majority of p53 null mice (and *Msh2* null) are viable has allowed questions to be posed addressing the importance of loss of gene function for phenotypically normal cells in vivo, when previously these questions had been restricted to (usually immortalised) cell lines in vitro. Although studies on such lines have produced invaluable data and will continue to do so, nearly all of these lines carry multiple genetic alterations. This makes the task of dissecting out the effect of individual genes very difficult.

The advent of knockout technology has not only produced a range of new in vivo systems, but it has also permitted the production of a new series of in vitro models. An obligatory step in the production of the knockout mice is the generation of targeted embryonic stem (ES) cells. If so desired, these can be rendered homozygous by either a second round of targeting or by the use of high levels of the appropriate antibiotic, which can select for conversion of the wild type allele. The availability of mutant cell lines are not, however, restricted to ES cells, as primary lines, such as embryonic fibroblasts (MEFs) can relatively easily be derived from mutant strains. Such primary lines carry the considerable advantage that they will not have been selected for additional genetic change in culture, and so should parallel the genotype of the mutant strain.

In each of these systems attempts have been made to determine the effect of genotype upon mutation rate. In reality these assays measure mutation frequencies, and broadly fit into two categories. First, those that that focus on a phenotypic change due to inactivation or overexpression of a somatic gene which is either autosomal or X-linked. These include changes resulting in loss of function at the *Dlb1* [37] and hypoxanthine phosphoribostltransferase (*HPRT*) loci [38], overexpression of metallothinein [39] and ouabain resistance [40]. The second category includes those approaches which measure mutation frequency from exogenous transgenes, including *lacI* (Big Blue^{TM}, *lacZ* (Muta^{TM}) and *supF* (tRNA suppressor gene) [40].

The most commonly used in vivo assays in the mouse are the *Dlb1* assay, and assays using the *lacI* and *lacZ* transgenes [41,42]. The *HPRT* is the most commonly used assay for in vitro estimates [38]. All of these techniques have drawbacks associated with them, however, it is encouraging that many of the in vivo tests yield relatively similar frequencies (see [43]).

The *Dlb-1* locus encodes for 2 lectin binding proteins. *Dlb-1a* is constitutively expressed on vascular endothelium. The *Dlb-1b* allele specifies binding of the Dolichos biflorus agglutinin to intestinal epithelium. Thus, in mice heterozygous for these alleles a single inactivating mutation at the *Dlb-1b* will abrogate the ability to bind this lectin in the intestinal epithelium. After cell proliferation and clonal expansion, mutations that occurred in the stem cell population will form clones that fail to bind a peroxidase conjugate of Dolichos biflorus agglutinin. These mutant clones can easily be visualised and scored to give an estimate of the mutation frequency.

As with the other approaches, the *Dlb-1* assay does suffer from some shortcomings. One drawback of this approach is that it is limited to the analysis of intestinal mutation rates, as specific *Dlb1-1b* expression is restricted to the intestine, and thus, mutation frequency can only be estimated in this tissue. A further difficulty is that the locus has not yet been cloned and so questions relating to mutational spectra cannot be addressed [44]. The assay does, however, possess a number of unique advantages over other approaches. Key amongst these is its ability to specifically score mutation within the stem cell population and to permit visualisation of the mutant clones in situ [41]. Furthermore, because the *Dlb-1* locus is an endogenous allele, there are few concerns over the in vivo relevance of results, as have been raised following the use of an exogenous transgene.

When mutation frequencies are compared between the *Dlb1* assay and the transgenic mice Big Blue^{TM} and MutA^{TM}, the transgenic models generally appear a little less sensitive [43]. You et al. [45] showed that one of the reasons for this was the presence of a large number of CpG sites in the *lacI* gene which are methylated in vivo. The *lacI* transgene usually consists of multiple concatemers (around 40) of the *lacI* gene within a λ-like shuttle vector. This can be harvested from the mouse genome and packaged within the λ phage. Bacteria are then infected on XGAL containing plates. After lysis those phages which contained

a mutated copy of the *lacI* will produce clear plaques whilst those with a functional copy will produce blue plaques. The number of blue plaques, therefore, provides an estimate of mutation frequency [42,44,45]. Likewise the transgene used in the MutA^{TM} system is *lacZ*, which encodes the β-galactosidase gene. Here the numbers of mutants are counted by growing the phages on PGAL (Sigma) containing substrate, which prevents wild type *lacZ* plaques from growing [46].

One feature of the transgenic assays is that they allow the precise nature of the mutation to be determined. This can, therefore, be used to generate mutation spectra, the detail of which can often be used to rationalise apparently contradictory results. For example, one unexpected finding was that mutation frequency in the *lacI* transgene did not increase significantly following treatment with X-rays, whilst a marked increase was observed at the *Dlb-1* locus [47]. The probable explanation for this is that X-rays predominantly cause gross deletions, which may result in deletion of the entire shuttle vector. In such circumstances these events will be under-represented in the *lacI* assay but not the *Dlb-1* assay.

One potential difficulty with assays using endogenous loci is that there may be in vivo selection against mutants. For example, it has been shown that there is selection against *HPRT* deficient T-lymphocytes cells in mice. Also, large deletions encompassing the *HPRT* locus can be selected against as a result of the deletion of flanking loci [48]. Notably, these difficulties have not been reported for the *Dlb-1* assay. Some of these considerations may also hold true for the transgene based assays, as the site of transgene integration is thought to influence assay readout [49].

5. Spontaneous mutation frequency in p53 deficient and *Msh2* deficient mice

Despite the individual limitations of each approach, the assays described above have been shown to yield broadly consistent results, and to be capable of identifying increases in mutation following most types of DNA damage. These assays have now been applied to murine strains mutant for genes implicated in DNA repair. Such analyses have proven particularly powerful where use has been made of both the transgenic and endogenous gene-based assays. For example, an increase in mutation frequency in vivo in the *Msh2* null mouse has been reported using both the Big Blue^{TM} mouse and *Dlb1* assay [50,51]. The Big Blue mouse showed an increase in all the three tissues studied: small intestine, thymus and heart. Comparison of the data generated by the two assays shows remarkable similarity: an approximate 10-fold increase was scored in the Big Blue mouse (3.1×10^{-5} in wild type intestine rising to 34×10^{-5} in the *Msh2* null intestine). A similar seven-fold increase was scored at the *Dlb-1* locus (four mutants per 10,000 villi for wild type intestine rising to 28 mutants per 10,000 villi in the *Msh2* nulls intestine). Furthermore, both assays concurred in that heterozygosity for *Msh2* did not impact upon mutation frequency.

The proposed role for p53 as a 'guardian of the genome' predicts that p53 deficiency should lead to an increase in mutation frequency. Surprisingly, this has not been substantiated experimentally [37,42,44,52]. The initial report [42] showed no increase in the spontaneous mutation frequency in the liver, spleen or brain of the Big Blue^{TM} mouse when bred onto a p53 null background. Subsequent sequencing of the entire *lacI* gene confirmed that there was no increase in either mutation frequency or spectra [44]. One possibility was that p53 deficiency only influenced the mutation rate in some tissue types. The most likely candidate tissue for such an effect was the thymus, as this is heavily predisposed to malignancy in the p53 null background. However, a similar study to those above extended the essentially negative finding to this tissue [53]. When spontaneous mutation frequency in the small intestine was examined at the *Dlb1* locus, again this yielded no significant p53–dependent increase. Likewise when Corbet et al. [38], examined mutation frequency at the *HPRT* locus in ES cells, p53 null ES cells again failed to show a significant elevation in mutation frequency.

Five potential explanations may be advanced to explain the observed lack of p53-dependency. First, these experiments were performed with 6–10 week old mice and it could be argued that insufficient time had elapsed for mutations to accumulate [42]. Certainly this is possible, if p53 is considered to be responding to DNA damage which will accumulate with age. However, since the majority of cells will have undergone many rounds of replication by 6–10 weeks of age, it seems unlikely that they will not have been exposed to substantial DNA damage [42].

Second, the spectrum of mutations identified by these assays may be inappropriate for the type of instability produced by lack of p53. However, this possibility seems extremely unlikely given the concordance between the different types of assay.

A third possibility arises from the fact that a large proportion of p53 mutations in human tumours are dominant negative, implying that the nullizygous state may be an inappropriate model for the study of p53 as a tumour suppressor [12]. However, the vast majority of studies strongly argue that loss of function of p53 is functionally equivalent, whether through the acquisition of a dominant negative mutation or through a homozygous null genotype. Although this difference is an important caveat to remember when considering the p53 null mouse as a model for p53 mediated tumourigenesis, it is extremely unlikely to bear any significance on the role played by p53 in protecting against mutation in normal cells.

A fourth possibility is that p53 is only relevant in guarding against increased mutation within abnormal or malignant cells. However, this also seems unlikely, as when Buettner et al. [53] analysed thymic lymphomas from p53 null mice only one out of four tumours had an increased mutation frequency compared to normal thymus. In four tumours examined by Sands et al. [52], none were found to have an increased mutation frequency. This in itself is a fascinating observation, questioning the mutator hypothesis of cancer which attempts to explain why tumours acquire so many somatic mutations (far above the postulated mutation rate for somatic tissues) (see [54]).

Finally, it may be that p53 cannot be shown to play a role because it is essentially redundant. Recently, several p53 homologues have been identified that could theoretically substitute for p53 in the p53 nulls. For example, the homologue p73 has been implicated in p53 independent apoptosis following both cisplatin treatment and ionising radiation [55,56]. Obviously, this cannot reflect complete redundancy, otherwise no exclusively p53-dependent phenomena (such as p53-dependent apoptosis) would be observed. However, given that mutation frequency is almost certainly determined by a number of interacting factors, redundancy remains a tenable explanation.

Given the current state of the field, it is not yet possible to discriminate between these possibilities. However, the fundamental observation that deficiency of p53 fails to influence spontaneous mutation rate has challenged the view that p53 plays a direct role that in DNA repair. It has also questioned the in vivo significance of p53 dependent apoptosis and cell cycle arrest.

6. Apoptosis, clonogenic survival and mutation frequency in vivo

The preceding discussion has addressed the consequences of loss of gene function at spontaneous levels of DNA damage. These studies are clearly limited to the low levels of environmental insult that may exist in a normal laboratory animal house setting, and will therefore, not address the consequences of exposure to defined types of DNA damage. Analysis using spontaneous levels of DNA damage also precludes the determination of a series of endpoints. Thus, apoptotic dependency can only be scored in circumstances that induce apoptosis. Similarly, clonogenic survival can only be scored when the majority of cells are lethally damaged. This is a particularly crucial endpoint to study as the emergence of malignant clones must be absolutely dependent upon the long term survival of the founder cell. For these reasons mice mutant for p53 and *Msh2* have been studied following exposure to defined types of DNA damage.

Amongst the first studies performed using the p53 knockout mice were those investigating the p53-dependency of apoptosis following genotoxic insult (normally ionising radiation). Initially Clarke et al. [57] and Lowe et al. [58] showed that unlike wild type thymocytes, which rapidly undergo apoptosis following treatment with ionising radiation and etoposide, p53 null thymocytes were resistant. Heterozygotes were found to have an intermediate phenotype. However, p53 null thymocytes were not resistant to the glucocorticoid methylprednisoline, nor to apoptosis induced following treatment with a calcium ionophore. These experiments showed that p53-dependence was restricted to certain types of stress, usually clastogenic damage. These observations gave rise to the notion that the failure to engage apoptosis may be the critical predisposing factor to tumourigenesis in a p53 null environment. Thus, cells exposed to DNA damage would not be deleted in the absence of p53, but would persist with a higher mutation burden, and therefore, a greater predisposition to malignancy.

Many different groups have attempted to directly test this hypothesis in vivo. As discussed above, p53 deficiency apparently failed to influence the spontaneous mutation frequency. In order to analyse the effects of p53 deficiency post DNA damage, Griffiths et al. [59] analysed short term primary cultures of IL7-dependent B cell precursors following exposure to X-rays. Here, they showed that p53 nulls were resistant to apoptosis and that there was no gross difference in DNA repair as scored by the COMET assay. Clonogenic survival was measured and the p53 null cells were found to have significantly enhanced survival. Mutation frequency was scored amongst the surviving cells, yielding an intriguing result. The frequency of mutants arising in the p53 null cells was found to be equivalent to that previously scored for wild type cells. However, the total mutation burden (number of mutant clones) was considerably higher than predicted for wild type cells as a direct consequence of the difference in clonogenic survival. Taken together, these findings strongly argue for a p53-dependent increase in mutation burden occurring through increased clonality. These results are therefore essentially supportive of the central hypothesis [3,59].

The in vivo p53-dependency of apoptosis has also been investigated in the small intestine and in splenocytes. Analysis of both these tissue types again identified a wave of p53-dependent apoptosis peaking at 4–6 h. However, a delayed p53-independent wave of cell death was also identified which was specific to the intestine and occurred at 48–72 h [37,60–62]. These results highlight clear tissue specific differences in the dependence upon p53 for the induction of apoptosis. When this p53 independent wave was examined further, it was noted that the apoptotic bodies were much larger, suggesting that cells had entered apoptosis from a G2 block (see [62,63]).

More recently, we have noted a p53 independent wave of apoptosis for two other cytotoxic drugs: temozolomide (an alkylating agent) and cisplatin [51,64]. The precise significance of p53-independent death remains to be established, however, it seems likely that it is effective in removing many of those cells survive by virtue of p53 deficiency. This may well reflect p73 activity, as has been reported in other systems [55,56].

The tissue specific differences in p53-independent death may also explain two basic observations made in the p53 null mice. First, the failure to see a p53-dependent difference in spontaneous mutation frequency in the intestine, as here there is an efficient alternative to p53-mediated death. Second, the strong predisposition to T-cell lymphoma in p53 null mice, as this tissue relies exclusively on p53 to mediate cell death.

7. Mutation and clonogenic survival following DNA damage in the intestine

The small intestine provides an extremely well characterised system in which to study cellular proliferation, apoptosis and differentiation (for a review see [65,66]). It is proposed that at the base of the crypt, there are approximately four to six stem cells. These are thought to be exquisitely sensitive to damage and undergo apoptosis under low levels of radiation (1 Gy). However once these are destroyed other clonogenic cells can then substitute for the original stem cells. Following exposures of up to 9 Gy, there are thought to be an additional six clonogenic cells. Beyond this level of damage it is thought that up to 16–24 cells can then act as clonogenic cells (approximately 1/3 of the crypt) [67].

The significance of the above is that clonogenicity can be investigated in vivo via an analysis of crypt survival (the micro-colony assay [68]) and this data can subsequently be related to the ability to engage apoptosis. One problem with this assay is that relatively high levels of DNA damage must be used to deliver scorable crypt death. However, this assay circumvents one of the major problems of in vitro assays in that it scores clonogenicity in a normal cellular setting.

Mutation frequency has also been investigated following exposure to DNA damage using those approaches discussed above. Again, the prediction from the central hypothesis is that p53 deficiency will result in a significant increase in mutation frequency following treatment. Both clonogenicity and mutation frequency experiments have generated similar data. Hendry et al. [69] showed that there was essentially no increase in survival in p53 null mice in the small intestine after gamma irradiation. In the large intestine, this study even indicated that p53 nulls were more sensitive than wild type counterparts. We have examined mutation frequency at the *Dlb1* locus Clarke et al. [37]. At 200 and 400 rads, there was no significant

difference between p53 nulls and wild types. However, p53-dependent difference was observed at the higher dose of 600 rads. Notably, this increase was not seen to be p53 gene-dose dependent, despite the fact that heterozygotes have an intermediate apoptotic phenotype.

One possible explanation for these results is that p53-independent apoptosis may be sufficient to remove cells harboring DNA damage cells following exposure to low doses of ionising radiation, but that this mechanism is incapable of dealing with the damage inflicted at high doses. Such an explanation does not, however, rest easily with the failure to see a p53-dependent difference in clonogenic survival, as this predicts a difference at the high levels of DNA damage used in the reported assays.

It is also quite possible that the nature of p53-dependency will alter with other insults. For example, we have obtained data indicating a clear p53-dependency on clonogenic survival following treatment with cisplatin [64].

In summary, the available data examining the relationship between apoptosis, clonogenic survival and mutation frequency in vivo is at the least inconsistent with the simple hypothesis originally proposed. Indeed, the clearest published demonstration of p53 dependency in clonogenic survival appears to disassociate apoptosis and clonogenicity [70]. Here, both low (40 mg/kg) and high (400 mg/kg) doses of the drug 5 fluorauracil (FU) was used. Both induced similar levels of apoptosis, which was p53 dependent at the 24 h peak (although in the wild types the levels of apoptosis returned back to basal levels sooner in the animals treated at 40 mg/kg). However, it was only the mice treated at the higher dose which showed increased p53-dependent clonogenic survival. Pritchard et al. [70] suggested that this was due to changes in cellular proliferation as mitotic cell indices and thymidine incoroparation fell to a much lower level in the mice treated at the higher dose. Thus, the presence or absence of a p53 dependent growth arrest is suggested as the crucial factor governing clonogenic survival. These results again strongly suggest that simple correlations cannot readily be drawn between loss of apoptosis, increased clonogenic survival and mutation frequency.

The situation as described for p53 contrasts somewhat with studies performed on *Msh2* deficient mice treated with the alkylating agents temozolomide and MNU (*n*-methyl-*n*-nitrosourea) [50,51]. Both of these drugs produce the cytotoxic O^6 methylguanine lesion. This lesion generally produces a G–T mispair after replication which is recognised by the mismatch repair machinery. Karran and Bignami [71] proposed that as this is recognised as a mismatch, the MMR machinery will remove the newly synthesised T. However, there will be no base that will correctly pair with the O^6 methylguanine lesion so it is proposed that this causes cycles of abortive repair which will eventually lead to apoptosis [71]. Toft et al. [51], showed that in the small intestine, *Msh2* deficiency reduces the apoptotic response to both temozolomide and MNNG (5 methyl-*N'*-nitro-*N*-nitroguanidine). Unlike the situation with p53 deficiency, there was no delayed *Msh2*-independent death. When clonogenic survival and mutation frequency was investigated in vitro in *Msh2* null ES cells, these showed both an increase in survival and mutation frequency relative to wild type controls. Significantly, an element of the increase in mutation frequency could be attributed to increased clonogenic survival. When mutation frequency was measured in vivo at the *Dlb-1* locus, there was a significant dose dependent increase in mutation after temozolomide treatment. Andrew et al. [50] also showed a significant increase (above basal levels) in mutation using the Big Blue™ mouse after treatment with MNU (*N*-methyl-nitrosourea). Thus, here there seems to be a clear relationship between loss of apoptosis and increase in survival and mutant cells, indicating that loss of apoptosis could be important in predisposing to malignancy. However, it is worth noting that as with many studies of this type, the level of DNA damage used was far from physiological. This may be of particular relevance when studying alkylating agents, as many O^6 methylguanine lesions are normally removed in vivo by the action of ATase (O^6 alkylguanine transferase). It may be that defects in the mismatch repair machinery only become relevant when this pathway is saturated, for example as might occur following high dose chemotherapy [72].

8. P53 and tumourigenesis

From the above it is possible to conclude that although p53 is clearly important in inducing apoptosis

in normal cells, the significance of this upon long term survival and mutation rate remains unclear. It may be that there is indeed a simple relationship, but that it has been obscured because the assays for clonogenicity and mutation frequency are insufficiently rigorous. It however seems more likely that a relationship does exist, but that it is highly damage and cell type dependent. This raises the question of relevance of these pathways in morphologically abnormal and neoplastic cells.

Relatively little data has been produced to approach this question. Fazeli et al. [73] investigated the induction of apoptosis within intestinal adenomas arising in either a wild type or p53 null background. They reported that the p53-dependent apoptotic response remained intact within adenomas following gamma irradiation. However, a failure to observe similar p53 dependency has been reported in neoplasia arising in p53(−/−)/MMTVras and p53null/Wnt1 mice [74].

In contrast, a number of other studies have associated the loss of an apoptosis programme with tumour progression. For example, use of an SV40 T antigen mutant capable of binding *Rb* but not p53 resulted in tumour development that was slower than mice expressing wild type T antigen. This difference disappeared when these mice were subsequently crossed onto a p53 null background [75]. Significantly in p53 heterozygotes, loss of heterozygosity correlated with a reduction in apoptosis and increased tumourigenesis. In further support of a role for apoptosis in mediating tumour suppression, similar results were obtained when the mutant T antigen was crossed onto a *Bax* mutant background (a downstream apoptosis inducing protein transactivated by p53) [76].

A role has also been demonstrated for p53 in mediating apoptosis in response to hypoxia [77]. This is of particular relevance to tumour development, as regions of low oxygen and necrosis are common features of solid tumours. Loss of this apoptotic pathway has therefore been proposed as a key feature in tumour development and in the selection within tumours of more aggressive clones.

Co-operativity between different mutations has been studied using mice bearing multiple germline mutations (for review see [16]). For example, p53 knockouts crossed onto *Msh2* $^{-\backslash -}$, *Rb*$^{+/-}$, APC$^{+/-}$, ATM$^{-\backslash -}$ mutant backgrounds all show enhanced tumourigenesis. In the case of p53$^{-\backslash -}$/APC$^{+\backslash -}$ mice this caused an increase in pancreatic neoplasia but had no effect on intestinal neoplasia [77]. This finding is consistent with a role for p53 deficiency late in intestinal tumourigenesis, but clearly does not support the notion that loss of a p53-dependent apoptotic pathway predisposes to neoplasia. Furthermore, these results challenge the significance of apoptotic pathways in tumour prevention within the pancreas, as normal pancreatic acinar cells have very low levels of apoptosis compared to the intestine [78].

The observation that p53 deficiency predisposes to a tissue specific pattern of tumourigenesis underlines a cell-type specific role for this protein, which may be interpreted in the light of the multiple roles attributed to p53. Thus, in some tissues loss of an apoptotic pathway may be crucial, whilst in other tissues loss of a cell cycle checkpoint or altered differentiation status may be the key predisposing factors [79].

Although we can, therefore, associate loss of p53 function with neoplasia, the relationship is clearly complex and imperfectly understood. One reason for this may be that studying mice characterised by complete loss of p53 function is simply too complex. To this end, a significant effort is now being is focussed on specific p53 mutants that are seen in neoplasia [80]. For example when p53^{172H} was expressed in the epidermis of mice no increase in neoplasia was seen [81].

However, an increased susceptibility to chemical carcinogenesis was observed. The development of these models and animals with engineered tissue specific knockouts of p53 (for example to avoid early onset lymphomagenesis) will undoubtedly provide better insights into the functional roles played by p53 in tumour suppression.

9. Conclusions: are we any closer to understanding the in vivo significance of *p53*?

The generation of p53 null mice has provided an excellent in vivo model to test hypotheses formed from clinical data and cell culture experiments. A number of these have been borne out. As predicted, p53 deficiency was found to predispose strongly to neoplasia, although the tumour spectrum did not accurately mirror tumourigenesis in humans. P53 deficiency has also been demonstrated to lead to loss of the apoptotic

response to DNA damage, as predicted from in vitro studies. However, experiments using the p53 null mice have identified a number of surprising results. Perhaps the first of these was that the null animals survived to birth at all, given the widespread roles proposed for p53. A partial role for p53 in embryogenesis has now been established. Other results have directly contradicted predictions from experimental systems. After genotoxic insult, despite the presence of a wave of p53-dependent apoptosis, increases in clonogenic survival and mutation are only seen in some systems and in some circumstances. Thus, for both of these phenomena there are tissue and drug specific variations in p53-dependency, and the significance to neoplasia of p53-dependent in vivo apoptosis remains to be proven.

These findings contrast somewhat to those obtained from mice deficient in the mismatch repair protein *Msh2*, which show clear increases in mutation following exposure to alkylating agents. ES cells mutant for *Msh2* also show increased clonogenic capacity, at least when scored in vitro. These increases are consistent with the observed loss of an *Msh2*-dependent apoptotic pathway, although the relative importance played by *Msh2*-dependent repair and *Msh2*-dependent apoptosis remains to be established in vivo [51].

It is also now clear that p53-independent pathways exist which can mediate the delayed induction of apoptosis in the absence of p53, and that these may be mediated by p53 homologues. It may be that only when these other pathways are fully characterised will we be able to determine the true relevance of cell death to neoplasia. For example, we have been able to show that loss of both *Msh2* and p53 causes complete loss of the apoptotic response following treatment with temozolomide [51]. This observation predicts a number of phenomena that can be directly tested, for example that treatment of an *Msh2* null tumour with an alkylating agent, such as temozolomide will select for loss of p53 (and vice versa).

In summary, the generation of p53 knockout mice has yielded a vast body of data on p53 function in vivo, and yet it may have left us with many more questions than answers. The original supposition that tumour suppression would be simply mediated by the apoptotic deletion of DNA damaged cells now seems somewhat naive. We, therefore, remain faced with the task of unravelling the in vivo relevance of each different aspect of tumour suppressor activity, both for p53 and its related genes. This process will be greatly facilitated by the rapid advancement in our understanding of the molecular pathway of p53 in vitro and by the generation of more advanced transgenic models.

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In vivo administration of O^6-benzylguanine does not influence apoptosis or mutation frequency following DNA damage in the murine intestine, but does inhibit P450-dependent activation of dacarbazine

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Clinically relevant cancer chemotherapeutic alkylating agents such as temozolomide and dacarbazine induce apoptosis and are mutagenic via the formation of O^6-alkylguanine adducts in DNA. The DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) functions by dealkylating such adducts and can thus prevent apoptosis and mutagenesis. In attempts to maximize the clinical effectiveness of these alkylating agents, inhibitors of AGT such as O^6-benzylguanine (BeG) have been developed. We show here that within murine small intestinal crypt cells, BeG administration does not alter the apoptotic response to the direct-acting methylating agents *N*-methyl-*N*-nitrosurea (MNU), temozolomide and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Furthermore, we show that BeG pretreatment fails to elevate the mutation frequency at the murine *Dlb-1* locus following exposure to MNU. Consistent with these results, we show that intestinal AGT activity is effectively abolished by administration of 100 mg/kg temozolomide, even in the absence of BeG. In contrast, pretreatment with BeG transiently abolished the apoptotic response to the methylating prodrug dacarbazine. Activation of dacarbazine to its reactive intermediate has previously been shown to be cytochrome P450 dependent and we show here that pretreatment of mice with the cytochrome P450 inhibitor metyrapone also inhibits dacarbazine-induced apoptosis. Thus BeG increases neither the prevalence of apoptosis nor mutation frequency in the murine small intestine, but is capable of inhibiting P450-dependent prodrug activation. The positive implication from this study is that BeG treatment may not exacerbate the toxic and mutagenic effects of methylating agents within normal cells, although it may engender other adverse reactions through the suppression of cytochrome P450-dependent processes.

Introduction

The murine small intestine offers an ideal experimental system to study both apoptosis and mutation frequency in response to DNA damaging agents such as γ-irradiation and alkylating agents (1–3). The DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) is expressed in the murine small intestine and is up-regulated in a *p53*-dependent manner in response to DNA strand breaks following ionizing irradiation (4). AGT functions by recognizing and removing specific alkyl lesions from DNA (5). The principal substrate is O^6-methylguanine, which is the major toxic and premutagenic lesion induced by methylating agents. AGT-mediated repair occurs via transfer of the alkyl group to a cysteine residue in the AGT protein, a process which is stoichiometric and autoinactivating (6). AGT therefore confers protection against the mutagenic and toxic effects of alkylating agents, which include the clinically important antitumour agents temozolomide and 5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide (dacarbazine) (7–10). Failure of AGT to repair O^6-methylguanine results in O^6-methylguanine:thymine mispairs following DNA replication and it is postulated that these mediate cell death via mismatch repair (11). The DNA mismatch repair enzyme MSH2 is known to bind to and recognize O^6-methylguanine:thymine mispairs and is also essential for a large proportion of apoptosis observed in the murine small intestine following exposure to alkylating agents (12,13). Mutations arise if the O^6-methylguanine:thymine mispairs undergo further rounds of replication.

O^6-benzylguanine (BeG) is a competitive and irreversible inhibitor of AGT which acts by binding to the $-CH_3$ cysteine acceptor site of AGT forming *S*-benzylcysteine (14,15). BeG-bound AGT is inactive and is subsequently degraded. Cellular AGT activity can be restored only by *de novo* protein synthesis (5) and its depletion by BeG has been proposed as a useful adjuvant to clinical methylating and chloroethylating agent treatment of tumours (16). Adjuvant therapy of BeG combined with such agents offers the potential to use lower, and hence less toxic, doses of chemotherapeutic drug and may also be of benefit in overcoming tumour resistance associated with high levels of AGT (17). Dacarbazine is a DNA alkylating agent used in the treatment of metastatic melanoma and Hodgkin's lymphoma (18). It is a prodrug requiring cytochrome P450-dependent *N*-demethylation to produce the active compound 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MITC) (19,20). MITC is also generated when temozlomide reacts spontaneously with H_2O (21). MITC decomposes to a methylating intermediate common to a variety of agents that result in the methylation of DNA with subsequent consequences for cell death and mutation.

One approach to directly determine the somatic *in vivo* mutation frequency relies upon detecting loss of function at the polymorphic genetic locus *Dlb-1* (22). This locus determines the ability to bind the lectin from *Dolichos biflorus* in mouse intestinal epithelium. Mutation frequency can be scored by identifying clonal populations which are no longer capable of binding the lectin. We have previously shown that *in vivo* administration of BeG reversibly suppresses AGT function, but does not modulate the apoptotic response to temozolomide

Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; BeG, O^6-benzylguanine; DBA, *Dolichos biflorus* agglutinin; dacarbazine, 5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide; MITC, 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosurea; PBS, phosphate-buffered saline.

or another methylating agent, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), in the small intestine. Furthermore, we have shown that BeG treatment did not enhance the apoptotic response in a mismatch repair-deficient background (13). We show here that AGT plays no role in modulating the apoptotic response or mutation rate in the normal epithelium of the murine intestine following exposure to the methylating agent *N*-methyl-*N*-nitrosurea (MNU) or the apoptotic response to cisplatin or γ-irradiation, but that it blocks dacarbazine-mediated cell death.

Materials and methods

Mouse colonies

All mice were maintained under non-barrier conditions and given a standard diet and water *ad libitum*.

AGT assay

Mice were killed and tissues of interest removed, snap frozen in liquid nitrogen and stored at −70°C until assayed as described previously (4). Briefly, tissue samples were defrosted and disrupted by sonication (10 s at 10 μm peak-to-peak, followed by 10 s at 16 μm peak-to-peak) in 1 ml of 50 mM Tris–HCl (pH 8.3), 1 mM EDTA, 3 mM dithiothreitol containing 5 μg/ml leupeptin, after which 10 μl of phenylmethylsulphonyl fluoride (87 μg/ml in ethanol) was added. Insoluble cellular debris was removed by centrifugation for 10 min at 13 000 r.p.m. at 4°C. Aliquots of the supernatants were incubated for 2 h at 37°C with a calf thymus DNA substrate that had been methylated by reaction with [^{3}H]MNU (14.5 Ci/mM; Amersham). Specific activities were calculated from the amount (fmol) of [^{3}H]methyl groups transferred per unit amount of total protein in the extract under conditions where activity was proportional to the amount of total protein assayed. The protein concentrations of the extracts were determined using bovine serum albumin as a calibration standard.

Treatment protocol

Eight to 10 week old mice were given i.p. injections of temozolomide (100 mg/kg), cisplatin (10 mg/kg; David Bull Laboratories), dacarbazine (150 mg/kg; Sigma), MNNG (50 mg/kg) or MNU (100 mg/kg). BeG was administered at a dose of 60 mg/kg i.p. Metyrapone was used at a concentration of 100 mg/kg i.p. The delivery volume for all reagents was 0.25 ml. All drugs, except cisplatin, were prepared fresh and first dissolved in dimethylsulphoxide (10% v/v) and made to a final concentration with phosphate-buffered saline (PBS) (temozolomide, dacarbazine and benzylguanine) or corn oil (MNNG). Metyrapone was dissolved in PBS alone. Mice were exposed to γ-irradiation using a ^{137}Cs source at 0.27 Gy/min for 15 min, so that each animal received a dose of 4 Gy.

Apoptosis quantitation

At a specified time point following reagent injection or irradiation, a minimum of three animals were killed, the small intestine removed, flushed with water and fixed overnight in methocarn (4 parts methanol, 2 parts chloroform, 1 part acetic acid). Haematoxylin and eosin stained sections were made and apoptosis scored through the use of the Highly Optimised Microscope Environment (HOME) as previously described (23,24). A minimum of fifty half-crypts were scored per animal.

Dlb-1 mutation assay

A cohort of 8–10 week old *Dlb-1* b/a heterozygote mice were treated with BeG (60 mg/kg i.p.). A second identical cohort received no BeG. One hour after BeG administration mice were injected i.p. with a single dose of MNU. Twenty-one days later the mice were killed, their small intestines removed and flushed with water. Whole mount preparations of 15 cm of the intestine were stained with *Dolichos biflorus* agglutinin (DBA)–peroxidase as described by Winton *et al.* (22). Villus ribbons not staining with the DBA–peroxidase conjugate were scored using a Wild stereo microscope and the results presented as number of mutations per 10^4 villi.

Results

BeG does not induce apoptosis within the murine small intestine

As previously shown, administration of 60 mg/kg BeG functionally depletes AGT activity in the small intestine for a period between 1 and 12 h post-drug administration (13). AGT inhibition may result in increased cell death as a consequence of failure to repair endogenous alkylated DNA damage. To test this hypothesis, apoptosis was scored over a time course following BeG administration. Cohorts of mice were injected with 60 mg/kg BeG at time 0. Apoptosis was scored at 0, 3, 6, 12, 24, 48 and 72 h after injection. No increase in the incidence of apoptosis was observed up to 72 h following BeG injection (data not shown). During this time period we also assessed the levels of necrosis within the sample histologically, as it remained possible that BeG was eliciting cell death through mechanisms other than apoptosis. We saw no increase in the levels of necrosis.

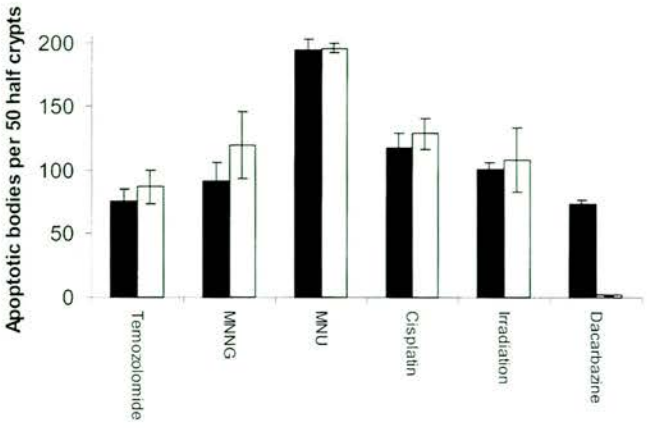

Fig. 1. The effect of BeG upon the induction of apoptosis in the murine intestine. Cohorts of wild-type mice were treated with 100 mg/kg temozolomide, 50 mg/kg MNNG, 100 mg/kg MNU, 10 mg/kg cisplatin, 4 Gy γ-irradiation and 150 mg/kg dacarbazine in the presence and absence of BeG. Data show the prevalence of apoptosis 6 h after reagent administration and 7 h after i.p. BeG (60 mg/kg). Each bar represents data from six mice. Closed boxes, no BeG; open boxes, BeG treated. Error bars represent SEM.

MNU-, cisplatin- and γ-irradiation-induced apoptosis in murine small intestine is unaffected by functional AGT depletion

We have previously shown that BeG administration did not modify enterocyte apoptosis following treatment with temozolomide or MNNG (13). We report here the effect of BeG administration on the apoptotic response to MNU, dacarbazine, cisplatin and γ-irradiation. These agents were chosen either to determine the effect of exposure to alkylating agents (MNU and dacarbazine) or to act as negative controls (cisplatin and γ-irradiation). Wild-type mice were pretreated with BeG (60 mg/kg) and 1 h later either 100 mg/kg MNU, 10 mg/kg cisplatin, 150 mg/kg dacarbazine or 4 Gy of γ-irradiation were administered. Comparative data on temozolomide- and MNNG-treated mice is reproduced here from Toft *et al.* (13). Apoptosis was scored in the small intestine 6 h later (Figure 1). No significant differences in the prevalence of apoptosis were observed between BeG- and non-BeG-treated animals following MNU, temozolomide, cisplatin or ionizing irradiation ($P > 0.4$ in all cases, Mann–Whitney *U*-test). However, dacarbazine-induced apoptosis was almost completely inhibited following BeG treatment ($P < 0.01$, Mann–Whitney *U*-test). Once again, we did not observe an increase in necrosis in any of the treatment protocols, indicating that these agents were not killing cells through mechanisms other than apoptosis, at least within the time frame studied.

The apoptotic response following low doses of temozolomide is not altered by BeG administration

To investigate the possibility that the doses of alkylating agents used in Figure 1 were depleting cellular pools of active AGT

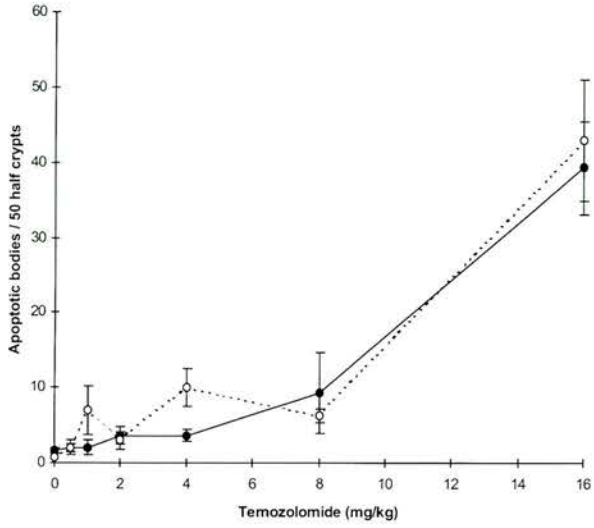

Fig. 2. The effect of BeG upon apoptosis induced by varying doses of temozolomide. Cohorts of wild-type mice treated with temozolomide (range 0.5–16 mg/kg) in the presence and absence of BeG. Data show the prevalence of apoptosis 6 h after temozolomide administration and 7 h after i.p. injection of BeG (60 mg/kg). Each point represents data from a minimum of three mice. Closed circles, BeG treated; open circles, no BeG. Error bars represent SEM.

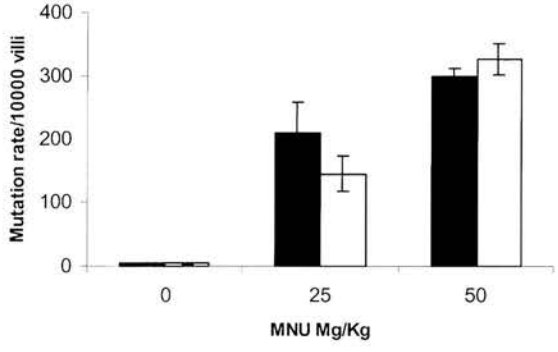

Fig. 3. The effect of BeG on mutation rates following exposure to MNU. *Dlb-1* mutation frequency in the murine small intestine following 100 mg/kg MNU treatment in the presence and absence of BeG. BeG was administered at a dose of 60 mg/kg prior to the administration of MNU. Each point represents data from three mice. Filled bars, no BeG; open bars, BeG treated. Error bars represent SEM.

and therefore rendering BeG treatment ineffective, very low doses of temozolomide (range 0.5–16 mg/kg) were administered to mice with and without BeG administration. Apoptosis was scored 6 h later (Figure 2). The rationale was that at low doses of temozolomide not all functional AGT activity would be abolished by the alkylating agent and thus, if AGT could actually alter the level of apoptosis, a difference between BeG-treated and non-treated mice may be revealed. No significant differences in the incidence of apoptosis were observed between BeG-treated and non-treated mice at any dose of temozolomide ($P > 0.07$ for all doses, Mann–Whitney *U*-test).

MNU-induced mutation frequency at the Dlb-1 locus is not influenced by BeG

Mutation rate within the murine small intestine following MNU was scored at the *Dlb-1* locus using standard approaches (1,22). The mutation frequency increased with increasing doses of MNU (Figure 3), but prior treatment with BeG did not result in a significant elevation in the mutation frequency at the *Dlb-1* locus ($P > 0.05$ for both doses, Mann–Whitney *U*-test).

Temozolomide administration effectively ablates AGT activity in the intestine

One explanation for the failure to observe BeG-dependent differences in apoptosis and mutation frequency is that treatment with the methylating agent alone is sufficient to render AGT functionally inactive and that, further, BeG-mediated suppression is therefore largely irrelevant. To directly test this we exposed mice to varying doses of temozolomide and scored AGT activity in both the liver and intestine. As we have shown before, pretreatment with BeG markedly reduces resting levels of AGT activity at 6 h (13). In the liver this reduction is from 150 ± 40.8 to 13.3 ± 21 fmol/mg protein. In the intestine AGT activity falls from a lower resting level (45.6 ± 12 fmol/mg) to become undetectable 6 h after BeG treatment (13). The effect of exposure to increasing doses of temozolomide was indeed to reduce levels of AGT activity, as shown in Figure 4. Following exposure to 100 mg/kg temozolomide, AGT activity was effectively depleted in both the liver and small intestine.

BeG inhibits the P450-dependent metabolic activation of dacarbazine

We have shown that exposure to BeG inhibits apoptosis induced by dacarbazine (Figure 1). In order to study the kinetics of this inhibition a single dose of BeG (60 mg/kg) was administered to wild-type mice at time 0. Mice were then subsequently injected with 150 mg/kg dacarbazine at 0, 1, 6, 12, 24, 48 and 72 h after BeG delivery. Six hours after each dacarbazine injection, levels of apoptosis were scored in the small intestine (Figure 5). Dacarbazine alone induced high levels of apoptosis with a mean of >60 apoptotic bodies per 50 half-crypts. However, between 1 and 6 h following BeG administration dacarbazine failed to induce apoptosis. This suppression was reversible, such that a normal apoptotic response was restored by 24 h. Thus, BeG administration reversibly inhibited dacarbazine-dependent apoptosis. The active DNA-damaging metabolite of dacarbazine is MITC, which is generated from dacarbazine in a cytochrome P450-dependent manner. In order to confirm that cytochrome P450 function was essential in eliciting a dacarbazine-dependent apoptotic response we used the P450 inhibitor metyrapone (25). Dacarbazine was administered 1 h following treatment with metyrapone (100 mg/kg i.p.). Metyrapone treatment significantly reduced the apoptotic response to dacarbazine (Figure 6), confirming that activation of the prodrug and the associated apoptotic response were P450 dependent ($P < 0.05$, Mann–Whitney *U*-test). These findings indicate that BeG administration blocks activation of the prodrug dacarbazine, possibly by compromising host P450 activity.

Discussion

We have directly addressed the ability of BeG to alter rates of apoptosis and mutation in the normal murine epithelium following exposure to a range of different types of damage. No induction of apoptosis was associated with BeG treatment. This excluded the possibility that either BeG itself or the depletion of AGT activity could result in the induction of apoptosis in the murine small intestine. Following treatment with BeG, and in the absence of detectable AGT activity, exposure to MNU, cisplatin and γ-irradiation all induced

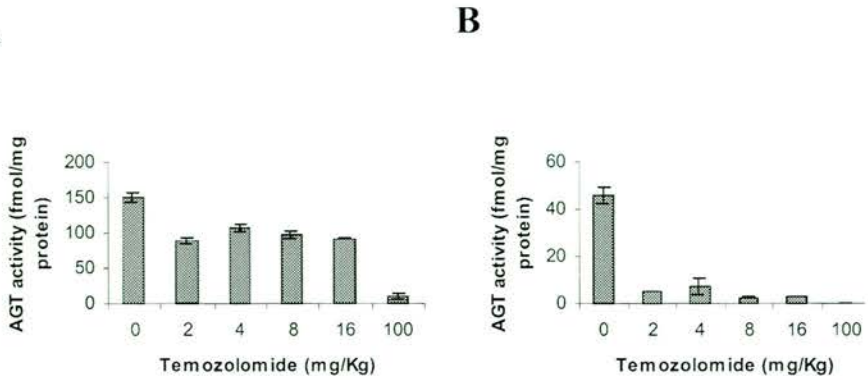

Fig. 4. The effect of temozolomide on AGT activity. AGT activity measured in fmol/mg protein scored in the liver (**A**) and in the small intestine (**B**) 6 h following a single i.p. injection of temozolomide. Resting values for intestine are reproduced from Toft *et al.* (13). Error bars represent SEM.

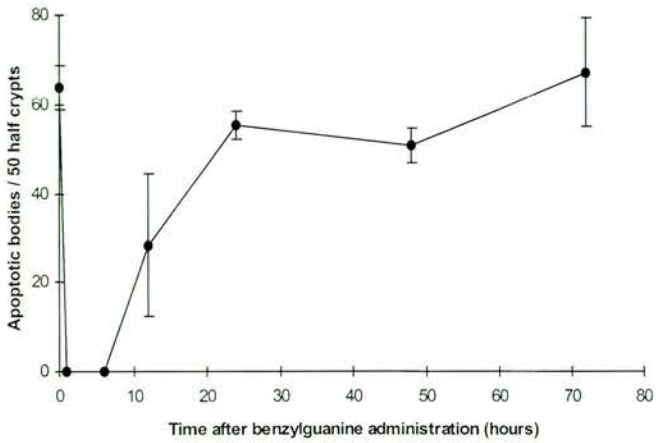

Fig. 5. The effect of BeG on dacarbazine-induced apoptosis. Following a single dose of BeG (60 mg/kg) at time 0, mice were injected with dacarbazine (150 mg/kg) at 0, 1, 6, 12, 24, 48 and 72 h after BeG. Six hours after each dacarbazine injection levels of apoptosis were scored in the small intestine. Each point represents data from three mice. Error bars represent SEM.

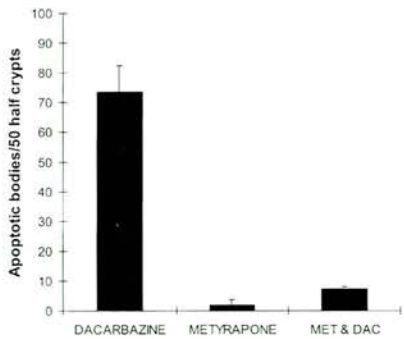

Fig. 6. The effect of metyrapone on dacarbazine-induced apoptosis. Bar chart of the prevalence of apoptotic bodies in the murine small intestine 6 h following 150 mg/kg dacarbazine or 100 mg/kg metyrapone. The prevalence of apoptotic bodies at 6 h in dacarbazine-treated mice which were pretreated with 100 mg/kg metyrapone 1 h prior to injection with dacarbazine is also shown (Met & Dac). Error bars represent SEM.

apoptosis. However, the levels of apoptosis were similar to those observed in BeG-untreated controls. Thus, BeG treatment neither reduced nor augmented the prevalence of apoptosis in the intestine following application of a variety of DNA-damaging agents.

Such a failure to modify the phenotype may have occurred because *in vivo* exposure to alkylating agents reduces AGT activity by depleting cellular pools of unmethylated AGT, so limiting the impact of BeG pretreatment (5,26,27). To address this possibility, temozolomide was administered at decreasing doses in the presence and absence of BeG. Similar levels of apoptosis were scored in both BeG-treated and BeG-untreated mice, suggesting that either ablation of AGT was occurring following exposure to very low levels of temozolomide or that BeG-mediated inactivation of AGT is irrelevant to the induction of apoptosis following alkylation damage. We further probed this question by analysing the effect of temozolomide administration upon AGT activity and established that low levels of temozolomide were effective in depleting AGT activity in the intestine. This is perhaps not surprising as resting levels within this tissue are relatively low. We also demonstrated a similar phenomenon for the liver, which is characterized by much higher basal AGT activity, although effective depletion was only seen at the highest temozolomide dose used, 100 mg/kg.

Increasing doses of MNU increased the mutation frequency scored at the *Dlb-1* locus. Since the principal mutagenic lesion generated by MNU is O^6-methylguanine, ablation of AGT activity might be predicted to lead to an increase in *Dlb-1* mutations. However, pretreatment of mice with BeG failed to elevate the mutation rate following MNU treatment. As with the effect of BeG treatment upon the induction of apoptosis, the observed failure to modify the mutation rate in the murine small intestine may be interpreted in several ways. First, AGT activity may not be relevant to protection against mutation in this tissue: other DNA lesions such as 3-methyladenosine could be responsible for these mutations. Second, exposure to the methylating agent alone may be sufficient to inactivate AGT, as suggested by our results following exposure to temozolomide. Third, it might be that in those cells in which *Dlb-1* mutations normally arise, recovery of AGT activity occurs prior to fixation of the mutation by DNA replication. It seems likely that the second of these explanations is correct, although it should be remembered that we have only established effective AGT depletion at a single time point for temozolomide. Taken together, these results show that BeG pretreatment does not alter either the apoptotic response or mutation frequency in the normal epithelium of the murine small intestine.

A somewhat unexpected finding in the present study was that BeG administration blocks dacarbazine-induced apoptosis. It is reasonable to suggest that this occurs either because AGT activity is required by dacarbazine to induce apoptosis or because BeG is blocking the normal metabolism of dacarbazine

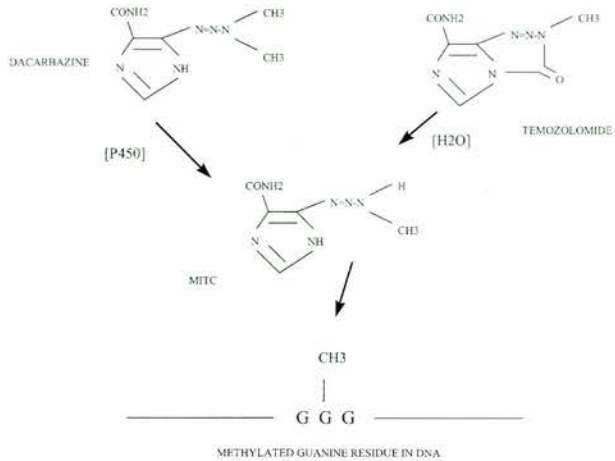

Fig. 7. Degradation pathways for dacarbazine and temozolomide. Dacarbazine and temozolomide give rise to the active compound MITC, which decomposes spontaneously to an active methylating agent which produces, amongst 11 other base lesions, O^6-methylguanine, the major premutagenic lesion in DNA. Dacarbazine requires oxidative *N*-demethylation by cytochrome P450 to form MITC, unlike temozolomide, which reacts spontaneously with water to give MITC.

to an active alkylating agent. Dacarbazine is known to undergo oxidative *N*-demethylation to its active compound MITC (18,28,29). This metabolic activation is mediated through the action of cytochrome P450 enzymes (20,30). Two observations argue in favour of the possibility that BeG is blocking the normal metabolism of dacarbazine. First, temozolomide is able to induce apoptosis in the presence of AGT depletion (13). Thus, the inhibitory action of BeG cannot be arising subsequent to the generation of MITC since it is also the active compound formed when temozolomide undergoes spontaneous chemical transformation (19,21) (Figure 7). Second, one of the major routes of metabolism of BeG in mice involves oxidation to form O^6-benzyl-8-oxoguanine (31), which occurs through the actions of aldehyde oxidase and the cytochrome P450 isoforms CYP1A2 and CYP3A4 (32,33). Dacarbazine is also metabolized by a number of different isoforms of P450, including CYP1A1, CYP1A2 and CYP2E1 (34). For both dacarbazine and BeG, the primary isoform responsible for metabolism is CYP1A2. The possibility therefore arises that BeG metabolism may competitively deplete cytochrome P450 activity. Thus, BeG appears capable of inhibiting at least some of the demethylating ability of cytochrome P450 enzymes in the mouse liver. The consequences of this novel finding are likely to be significant in view of the central role of P450 enzymes in the metabolism of drugs and carcinogens.

There is increasing interest in the use of BeG clinically as an adjuvant to alkylating agent chemotherapy to overcome tumour resistance mediated by AGT and to potentiate the cytotoxic effects of chemotherapy (16,35). There are, however, a number of uncertainties concerning the clinical benefits of BeG. First, alkylating agents themselves are capable of inactivating AGT activity by depleting cellular pools (5,27). We show here that this can be an effective mechanism for suppressing AGT activity both in the normal cells of the small intestine and in the liver of mice. There are, however, several lines of evidence to suggest that the use of alkylating agents to suppress AGT activity can be ineffective or even inappropriate, either because of failure to increase the therapeutic index or because of dose-limiting toxicity (27). Thus, although alkylating agents can reduce the activity of AGT, the available data indicate BeG administration to be the most effective and least toxic method to achieve this. Second, BeG has been reported to increase mutation rates, chromosome aberrations and toxicity in normal cells treated with methylating or chloroethylating agents (36–38). In contradiction to these reports, we do not observe any effect upon *in vivo* mutation rate in these studies. Third, from the data presented here it is clear that in the murine small intestine AGT depletion does not elevate *in vivo* apoptotic levels following methylating DNA damage. This result has clear positive implications for the therapeutic use of BeG, in that BeG administration fails to augment cell death within normal tissues. This result may directly follow from the demonstrated ability of alkylating agents to partially suppress AGT activity and may simply reflect the low resting levels of AGT in the murine intestine. Finally, and perhaps most significantly, these data show that BeG can alter the metabolism of drugs or carcinogens, exemplified here by activation of the prodrug dacarbazine, and may therefore be a cause of unexpected adverse drug reactions if used clinically.

Acknowledgements

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SHORT REPORT

A role for mismatch repair in control of DNA ploidy following DNA damage

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Many reports have shown a link between mismatch repair (MMR) deficiency and loss of normal cell cycle control, particularly loss of G2 arrest. However almost all of these studies utilized transformed cell lines, and thus the involvement of other genes in this phenotype cannot be excluded. We have examined the effects of cisplatin treatment on primary embryo fibroblasts (MEFs) derived from mice in which the MMR gene Msh2 had been inactivated ($Msh2^{-/-}$). This analysis determined that both primary $Msh2^{-/-}$ and wild type (WT) fibroblasts exhibited an essentially identical G2 arrest following cisplatin treatment. Similarly, we observed a cisplatin-induced G2 arrest in immortalized MMR deficient ($Mlh1^{-/-}$ and $Pms2^{-/-}$) and WT MEFs. p53 deficient primary MEFs ($p53^{-/-}$) exhibited both a clear G2 arrest and an increase in cells with a DNA content of 8N in response to cisplatin. When the *Msh2* and *p53* defects were combined ($p53^{-/-}/Msh2^{-/-}$) the G2 arrest was essentially identical to the $p53^{-/-}$ fibroblasts. However, the $p53^{-/-}/Msh2^{-/-}$ fibroblasts demonstrated a further increase in cells with an 8N DNA content, above that seen in the $p53^{-/-}$ fibroblasts. These results suggest that loss of MMR on its own is not enough to overcome G2 arrest following exposure to cisplatin but does play a role in preventing polyploidization, or aberrant DNA reduplication, in the absence of functional p53. *Oncogene* (2001) **20,** 1923-1927.

Keywords: mismatch repair; cell cycle; polyploidy; p53; DNA damage

DNA mismatch repair is known to play an important role in maintaining genomic stability, due to its function in correcting DNA mismatches introduced during replication, and cells deficient in MMR exhibit an increased mutation rate (for review see Buermeyer *et al.*, 1999). However the proteins that make up the MMR system have also been associated with a number of other phenotypes. Cells deficient in MMR have been shown to be resistant to a number of clinically important drugs, such as cisplatin, temozolomide and doxorubicin (Fink *et al.*, 1998). This resistance may be mediated by an altered ability to engage apoptosis, as the MMR proteins have been shown to be critical in mediating the normal *in vivo* apoptotic response to alkylating agents (Toft *et al.*, 1999). Restoration of MMR activity in cell lines, either by chromosome transfer or by direct re-introduction of the gene, results in drug resensitization, demonstrating that the drug resistance is not secondary to an increase in mutation rate but is due to the direct involvement of the MMR system. In addition MMR deficiency has also been associated with a loss of normal cell cycle controls in response to agents such as MNNG (Koi *et al.*, 1994), 6-thioguanine (6-TG) (Hawn *et al.*, 1995) cisplatin (Brown *et al.*, 1997) and ionizing radiation (IR) (Davis *et al.*, 1998), suggesting that the MMR system may have a regulatory role, primarily at the G2/M boundary. Indeed the activity of a number of key regulators of the cell cycle, such as p53 (Duckett *et al.*, 1999), p73 (Gong *et al.*, 1999) and c-*abl* (Nehme *et al.*, 1997), are known to be modulated in a MMR dependent fashion in response to certain types of DNA damage.

Loss of MMR has also been shown to play an important role in the development of cancer. Loss of at least five of the MMR proteins (MLH1, MSH2, MSH6, PMS1 and PMS2) has been shown to be associated with the hereditary cancer syndrome HNPCC (Buermeyer *et al.*, 1999). In addition loss of mismatch repair, and the resulting microsatellite instability phenotype, is seen in a significant fraction of sporadic tumours of many different cancer types (Arzimanoglou *et al.*, 1998). It is generally assumed that loss of MMR leads to cancer due to the resulting increased mutation rate, although it has also been suggested that defective cell cycle control may be the primary oncogenic mechanism in MMR deficient cells (Tomlinson and Bodmer, 1999).

Nearly all studies on the role of MMR in drug resistance and cell cycle control have been performed in tumour-derived or immortalized cell lines. Such cell lines will inevitably have multiple alterations in cell cycle control, which may well interact with a deficiency in MMR and influence the apparent phenotype of MMR defects. To more clearly define the role of MMR in the cell cycle response to a commonly used chemotherapeutic agent, cisplatin, we have assessed cell

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cycle responses in both primary and spontaneously immortalized mouse embryonic fibroblasts (MEFs) in which one of the mismatch repair proteins, Msh2, Mlh1 or Pms2, had been genetically inactivated. In addition we have also determined the response to cisplatin in p53 proficient and deficient backgrounds.

MEFs were isolated from *Msh2*$^{-/-}$ and WT embryos taken from 13-day pregnant females. After 3 days in culture the MEFs were split into 10 cm dishes to allow cell cycle analysis using BrdU/propidium iodide staining and FACS analysis. For each embryo, separate plates were treated with 0, 5 or 10 μm cisplatin for 1 h. Cells were then harvested at 24 or 48 h post-treatment for cell cycle analysis (example histograms in Figure 1). As expected the WT MEFs show a clear accumulation in G2 following exposure to

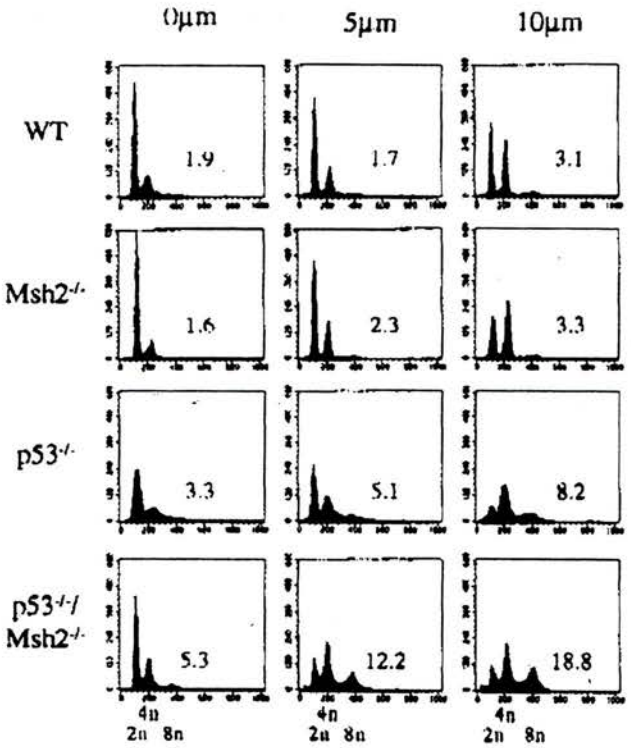

Figure 1 Effect of Msh2 and p53 status on G2 arrest and DNA ploidy. Representative FACS profiles are shown 48 h after treatment with 0, 5 or 10 μm cisplatin, as indicated, for MEFs of each of the four genotypes. The percentage of cells with a DNA content of 8N is shown on each profile. Primary MEFs were derived from 13-day-old embryos and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% foetal calf serum. After 3 or 4 days cells were trypsinized and approximately 1×10^6 cells were plated out for each dose/time point to be tested. The following day the cells were exposed to 0, 5 or 10 μm cisplatin (David Bull Laboratories) for 1 h at 37°C, then washed with PBS and fresh media added. Four hours before the cells were due to be harvested the media was removed and replaced with media containing 10 μm BrdU (Sigma) and incubated at 37°C for a further 4 h. The cells were then trypsinized, centrifuged and fixed in 70% ethanol. The fixed cells were stored at −20°C and flow cytometric analysis was performed as described before (Brown *et al*., 1993) using a FACScan (Becton Dickinson). Statistical analysis was performed using a two-tailed Student's *t*-test

5 or 10 μm cisplatin at both 24 and 48 h (Figures 1 and 2). The *Msh2*$^{-/-}$ MEFs also show a clear G2 arrest in response to cisplatin (Figures 1 and 2). Indeed, the G2 arrest seen in the *Msh2*$^{-/-}$ MEFs is essentially identical to that of the WT MEFs. This suggests that in these cells an intact MMR response is not required to initiate or maintain a G2 arrest following cisplatin damage. These results are in contrast to previous studies showing loss of G2 arrest following cisplatin treatment in MMR-defective cancer cell lines (Brown *et al*., 1997), and loss of G2 arrest after 6-TG or IR in MMR-defective MEFs (Davis *et al*., 1998). Taken together these results suggest that in primary MEFs a MMR defect is not sufficient to overcome G2 arrest in response to cisplatin, and that a MMR independent mechanism causes G2 arrest in response to cisplatin, but not 6-TG or IR. Further investigation will be required to determine the nature of the MMR-independent mechanism, however a number of other pathways leading to G2 arrest in response to DNA damage have previously been identified (Dasika *et al*., 1999).

Three spontaneously immortalized MEF cell lines, WT or deficient in either *Mlh1* (*Mlh1*$^{-/-}$) or *Pms2* (*Pms2*$^{-/-}$) (Prolla *et al*., 1998), were also used for cell cycle analysis. As for the primary MEFs the immortalized cell lines all exhibit a clear G2 arrest in response to treatment with 5 or 10 μm cisplatin (Figure 3). The G2 arrest observed in the *Mlh1*$^{-/-}$ and *Pms2*$^{-/-}$ immortalized MEFs is similar to that seen in the immortalized WT MEFs, although the results suggests that there is a more rapid exit from G2 arrest in the *Pms2*$^{-/-}$ MEFs (Figure 3; 10 μm/48 h time point). Therefore, as in the primary MEFs, a functional MMR system is not essential for the G2 arrest in response to cisplatin treatment.

The FACS analysis presented above does not differentiate between G2 cells and cells in mitosis. Therefore it is possible that the MMR deficient cells have indeed lost G2 arrest but then subsequently arrest in mitosis through a MMR independent mechanism. To address this, the mitotic index of the three immortalized MEF cell lines was determined in untreated and cisplatin treated cells. Experiments in which the mitotic spindle is disrupted by nocodozole treatment report a peak in the mitotic index at 8 h post treatment (Notterman *et al*., 1998). We therefore analysed cells at either 8 or 24 h after a 1 h exposure to 10 μm cisplatin. Cells were fixed in methanol : acetic acid (3 : 1) and dropped onto microscope slides. Mitotic cells were identified based on the presence of condensed chromatin. As can be seen from Table 1, there is no evidence of an increase in mitotic index in any of the three cell lines (indeed mitotic indices are reduced, presumably due to the arrest induced by cisplatin in other phases of the cell cycle) showing that arrest does not occur during mitosis. Our findings are therefore consistent with a G2 arrest both in the presence and absence of functional MMR.

The p53 protein is known to be a key regulator of cell cycle checkpoints and its function is frequently lost

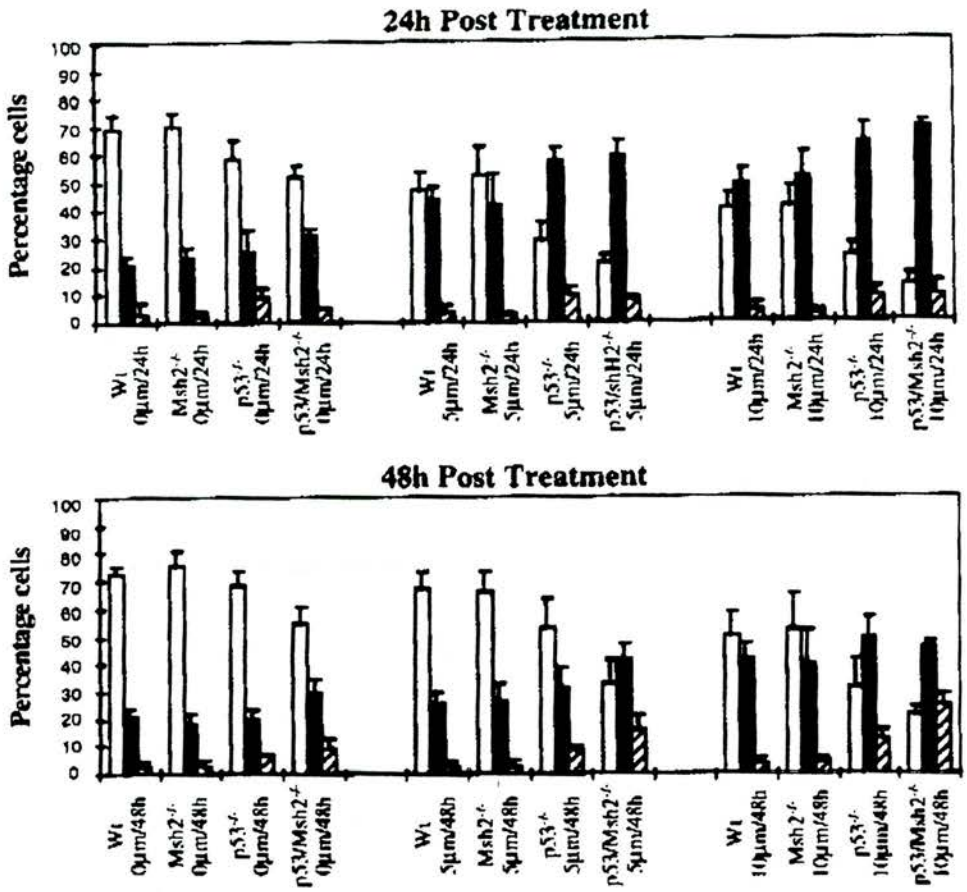

Figure 2 Effect of cisplatin treatment on cell cycle distribution of primary MEFs. The percentage of cells with a 2N, 4N or 8N DNA content was assessed by FACS analysis. Cells were treated with 0, 5 or 10 μm cisplatin for 1 h and their cell cycle distribution analysed either 24 or 48 h after drug treatment, as indicated. 2N cells are indicated by the open column, 4N cells by the filled column and 8N cells by the striped column. Each dose/time point is an average of 3–8 replicates. The standard deviation is represented by error bars

in human tumours (Bates and Vousden, 1996). In addition the absence of p53 has been shown to be associated with genomic instability and increasing anneuploidy (Donehower *et al.*, 1995). To further investigate the effect of loss of MMR on cisplatin induced cell cycle arrest we analysed primary MEFs deficient for both Msh2 and p53 and compared them with MEFs deficient for p53 alone using the same analysis as described above. As can be seen from Figure 2 the p53 deficient MEFs exhibit an even stronger G2 arrest than the WT MEFs. This is likely to be due to the loss of the p53 dependent G1/S checkpoint (El-Deiry *et al.*, 1993; Keurbitz *et al.*, 1992) resulting in more cells entering and then arresting in G2. As was seen in the WT background, the loss of Msh2 function in a p53 deficient background does not result in any loss of G2 arrest (Figure 2). A previous study of MMR deficient, cancer-derived cell lines, which did exhibit loss of G2 arrest in response to cisplatin (Brown *et al.*, 1997), also detected deficiencies in p53 function (Anthoney *et al.*, 1996). However, in this study we detected no loss of G2 arrest even when Msh2 was genetically inactivated in a p53 deficient background. Thus the MMR independent pathway that results in G2 arrest in response to cisplatin in primary MEFs is also able to function in the absence of p53 activity.

p53 is also thought to play a role in a second cell cycle checkpoint at the G2/M boundary to prevent aberrant reduplication of DNA (Cross *et al.*, 1995). Consistent with this observation our analysis detected an increase in cells with an 8N DNA content in the $p53^{-/-}$ MEFs compared with the WT MEFs (Figures 1 and 2), in both the cisplatin treated and untreated cells ($P = 0.028$, 0.0028, 0.0032 for 0, 5, 10 μm treated cells at 48 h post-treatment, respectively). The $Msh2^{-/-}$ MEFs showed no increase in the 8N population compared with WT MEFs. In contrast the $p53^{-/-}/Msh2^{-/-}$ MEFs showed a clear increase in cells with an 8N DNA content, over and above that seen in the MEFs deficient in p53 alone (Figures 1 and 2). This increase in 8N cells was observed in untreated cells ($P = 0.023$), as well as following treatment with either 5 ($P = 0.025$) or 10 μm ($P = 0.0075$) cisplatin at 48 h post-treatment. The results for the $p53^{-/-}$ MEFs are not unexpected, but the increased rate of polyploidy

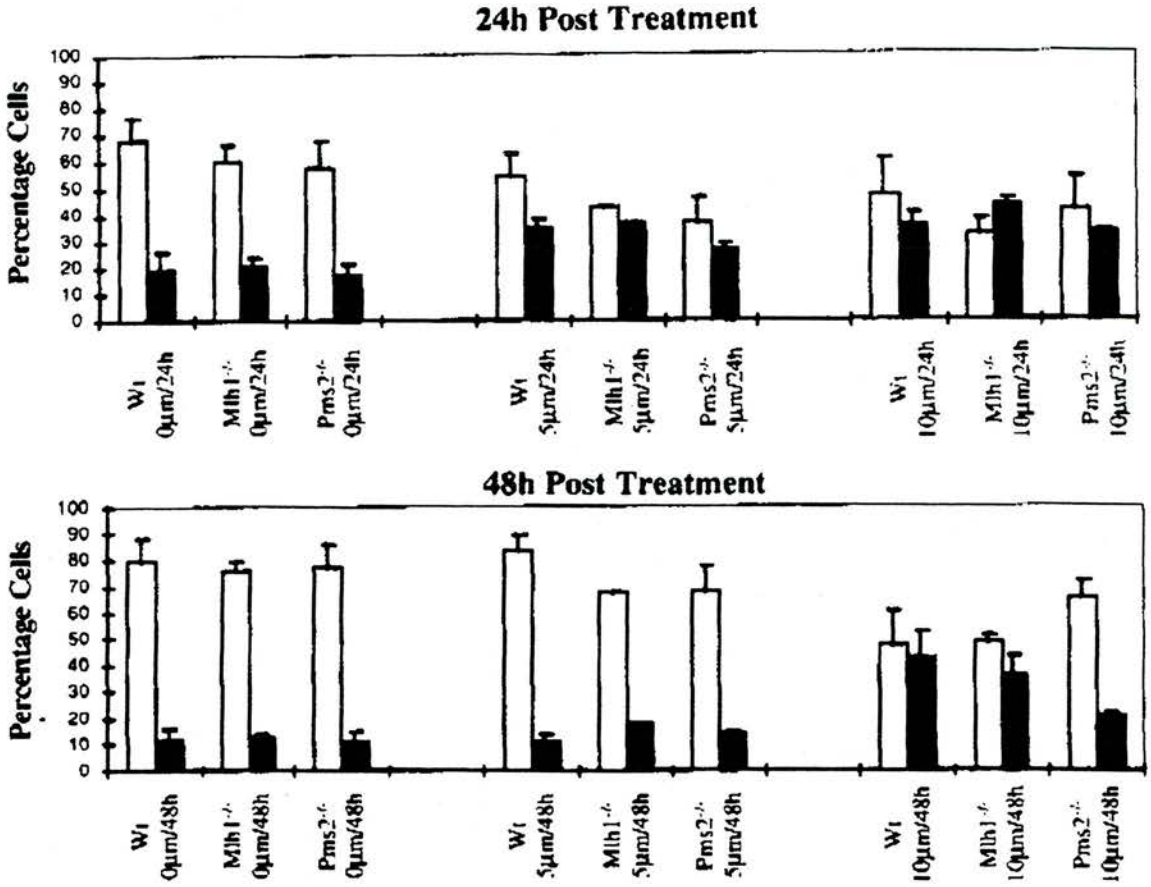

Figure 3 Effect of cisplatin treatment on cell cycle distribution of immortalized MEFs. The percentage of cells with a 2N or 4N DNA content was assessed by FACS analysis. 2N cells are indicated by the open column and 4N cells by the filled column. Each dose/time point is an average of four replicates. The standard deviation is represented by error bars

Table 1 Percentage of cells in mitosis

Genotype	*0 h*[a]	*8 h*	*24 h*
WT	2.10[b]	1.26	1.28
Mlh1$^{-/-}$	1.48	0.55	0.30
Pms2$^{-/-}$	1.67	0.90	1.22

[a]Time in hours afer exposure to 10 μm cisplatin at which cells were harvested. 0 h indicates untreated. [b]Percentage of mitotic cells

observed in the *p53*$^{-/-}$/*Msh2*$^{-/-}$ MEFs suggests a novel role for Msh2, and therefore possibly for MMR, in the prevention of aberrant reduplication of DNA, particularly in response to cisplatin induced DNA damage. Further investigation will be required to determine if Msh2 also plays a role in preventing aberrant reduplication of DNA in response to other DNA damaging agents, such as 6-TG and IR. There are a number of potential mechanisms by which Msh2 could function to prevent aberrant reduplication. Msh2 and its heterodimeric binding partner Msh3 are known to be required for removal of non-homologous tails during recombination (Sugawara *et al.*, 1997). It is therefore possible that MMR proteins could be involved in signalling from unresolved or aberrant recombination structures, to allow the resolution of such structures and the appropriate completion of mitosis. In the absence of MMR and p53 such cells may not be able to complete mitosis due to the MMR defect and may then aberrantly re-enter S phase due to the defect in p53 function. Interestingly, cisplatin treatment is known to increase mitotic recombination, as judged by an increase in sister chromatid exchange, a process regulated in part by MMR proteins (Durant *et al.*, 1999), and the Msh2 dependent increase in the 8N population is exaggerated following cisplatin treatment. Alternatively, Msh2 could act to induce apoptosis in polyploid cells, thus eliminating them from the population. A role for MMR proteins in signalling apoptosis in response to DNA damage is already well established (Hickman and Samson, 1999; Gong *et al.*, 1999).

In summary, our results highlight an interdependence between the MMR and the p53 pathways. Such an interaction is supported by a number of other studies. Lee *et al.* (1995) showed that p53 can recognize insertion deletion loops, forming stable complexes at these lesions where it can physically interact with Msh2. p53 has also been shown to suppress non-homologous recombination by specific mismatch

recognition (Dudenhoffer *et al.*, 1998), and MMR-dependent phosphorylation of p53 has been reported after methylation damage (Duckett *et al.*, 1999). Finally, combined loss of Msh2 and p53 has been shown to lead to embryonic lethality of female mice on a C57BL6J background and to synergistically increased tumorigenesis in males (Cranston *et al.*, 1997).

Previous studies obtained from tumour cell lines have implicated the MMR proteins in G2 arrest. However we show here that primary cell lines (and indeed immortalized lines derived from primary cell lines) do not show an MMR dependent G2 arrest following cisplatin treatment. These results demonstrate that MMR dependent responses are highly context dependent and that the processes controlled by the MMR proteins in normal and neoplastic cells can markedly differ. We also identify a new role for MMR proteins in controlling ploidy status, and a clear interaction in this process with p53. Taken together these results have important implications for our understanding of MMR function in normal and neoplastic cells.

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Dysregulated expression of β-catenin marks early neoplastic change in Apc mutant mice, but not all lesions arising in Msh2 deficient mice

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We have analysed the pattern of β-catenin expression by immunohistochemistry in mice singly or multiply mutant for *Apc*, *p53* and *Msh2*. We observed increased expression of β-catenin in all intestinal lesions arising on an $Apc^{Min}+/-$ background. In all categories of lesion studied mosaic patterns of β-catenin expression were observed, with the proportion of cells showing enhanced expression decreasing with increasing lesion size. p53 status did not alter these patterns. We also show that β-catenin dysregulation marks pancreatic abnormalities occurring in $Apc^{Min}+/-$ and ($Apc^{Min}+/-$, p53$-/-$) mice. In these mice both adenomas and adenocarcinomas of the pancreas arose and were characterized by increased expression of β-catenin. We have extended these analyses to intestinal lesions arising in mice mutant for the mismatch repair gene *Msh2*. In these mice, increased expression of β-catenin was again observed. However, in contrast with $Apc^{Min}+/-$ mice, a subset of lesions retained normal expression. Taken together, these findings show that increased expression of β-catenin is an efficient marker of early neoplastic change in both murine intestine and pancreas in *Apc* mutant mice. However, we also show that dysregulation of β-catenin is not an obligate step in the development of intestinal lesions, and therefore that genetic events other than the loss of Apc function may initiate the transition from normal to neoplastic epithelium.

Keywords: β-catenin; Apc; p53; Msh2; intestine; pancreas

Introduction

Germline mutations in the adenomatous polyposis coli gene (APC) gene at 5q21 characterize an inherited disorder known as familial adenomatous polyposis coli (FAP) (Kinzler *et al.*, 1991). FAP patients develop numerous adenomas throughout both the small and large intestine, some of which ultimately progress to carcinoma. However, a more general role for APC mutations in neoplasia is suggested by the fact that FAP patients have an increased predisposition to tumours of the brain, thyroid and bone and also to focal proliferative lesions ('desmoid tumours') of the connective tissue. Mutated APC has also been reported in a range of sporadic tumours, including pancreatic and gastric tumours and the majority of adenomas and carcinomas of the colorectum (Miyoshi *et al.*, 1992; Horii *et al.*, 1992; Nakatsuru *et al.*, 1992). Furthermore, loss of heterozygosity at 5q21 has been observed in sporadic tumours of the breast and oesophagus (Boynton *et al.*, 1992; Thompson *et al.*, 1993; Kashiwaba *et al.*, 1994).

Several different murine models of FAP have been generated by random chemical carcinogenesis (Moser *et al.*, 1992), conventional gene targeting (Fodde *et al.*, 1994; Oshima *et al.*, 1995) and by the use of Cre-Lox technology (Shibata *et al.*, 1997). All of these models are characterized by high levels of spontaneous intestinal neoplasia, confirming a role for *Apc* in the development of these lesions. Several observations using these models support the notion that *Apc* has more widespread tumour suppressor activity. First, desmoid tumours have been reported to occur spontaneously in *Apc* mutant mice (Shoemaker *et al.*, 1997; Smits *et al.*, 1998). Second, Apc^{Min} heterozygotes show an increased susceptibility to mammary carcinoma both spontaneously and following genotoxic stress such as carcinogen treatment or X irradiation (Moser *et al.*, 1993, 1995; van der Houven *et al.*, 1997). Finally, *Apc* heterozygosity on a *p53* null background has been shown to strongly predispose to pancreatic neoplasia (Clarke *et al.*, 1995).

How loss of function of Apc predisposes to malignancy remains unclear, however disruption of the normal function of β-catenin has been implicated in this process (Rubinfeld, 1993; Su *et al.*, 1993). Levels of β-catenin are modulated by Apc through the mammalian Wnt signalling pathway, where Apc interacts with both glycogen synthase kinase 3β (GSK3β) and β-catenin. The central portion of Apc contains sites at which it can be phosphorylated by GSK3β and also through which it complexes with β-catenin. Phosphorylation by GSK3β increases the stability of the Apc/β-catenin complex and is thereby thought to increase the rate of β-catenin degradation (Rubinfeld *et al.*, 1996).

From the above it is clear that disruption of Apc function can lead to an increase in the cellular levels of β-catenin. However, this is not the only potential mechanism for such an increase. Wnt-1 has been shown to regulate free pools of catenin (Papkoff *et al.*, 1996) and both axin and the axin homologue conductin have been reported to alter β-catenin activity through interaction with Apc, β-catenin and GSK3β (Behrens *et al.*, 1998; Ikeda *et al.*, 1998; Kishida *et al.*, 1998). The potential relevance of increased levels of β-catenin becomes clear in the light of findings which show that β-catenin functionally interacts with and activates members of the Tcf family of DNA binding

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transcription factors, including both Lef-1 and Tcf 4 (Behrens *et al*., 1998; Korinek *et al*., 1997). Activation of transcriptional signalling by β-catenin-Tcf complexes has been shown to occur as a consequence of mutations in both *Apc* and β-catenin (Morin *et al*., 1997; Rubinfeld *et al*., 1997) and mutations of β-catenin have been reported in human colorectal cancers (Sparks *et al*., 1998). Dysregulated transcription has therefore been proposed as the basis for early neoplastic change, although the target genes through which this may be mediated remain as yet undetermined (Nusse, 1997).

β-catenin also regulates E-cadherin in conjunction with α-catenin, and loss of function of any of these proteins abrogates E-cadherin activities, including maintenance of the adherens junction complex. Amongst other activities this complex mediates cell-to-cell adhesion and thereby the control of cell motility (Chen *et al*., 1997). Modulation of cell adhesion appears to be a common mechanism in neoplastic change, and altered E-cadherin activity has been found in a number of cancers of epithelial origin including lobular breast carcinoma, colorectal carcinoma and gastric adenocarcinoma (Birchmeier and Behrens, 1994). In a transgenic murine model of pancreatic β-cell carcinogenesis, loss of function of E-cadherin has been shown to be a critical step in the transition from adenoma to carcinoma (Perl *et al*., 1998).

Immunohistochemical analysis of both human and murine intestinal tumours has shown that both adenomas and well differentiated carcinomas are characterized by high levels of β-catenin (Inomata *et al*., 1996; Takayama *et al*., 1996). However, both β-catenin and E-cadherin are reported to be expressed at significantly lower levels in more aggressive malignancies, strongly suggesting that over-expression of β-catenin is only crucial in early tumour development (Takayama *et al*., 1996).

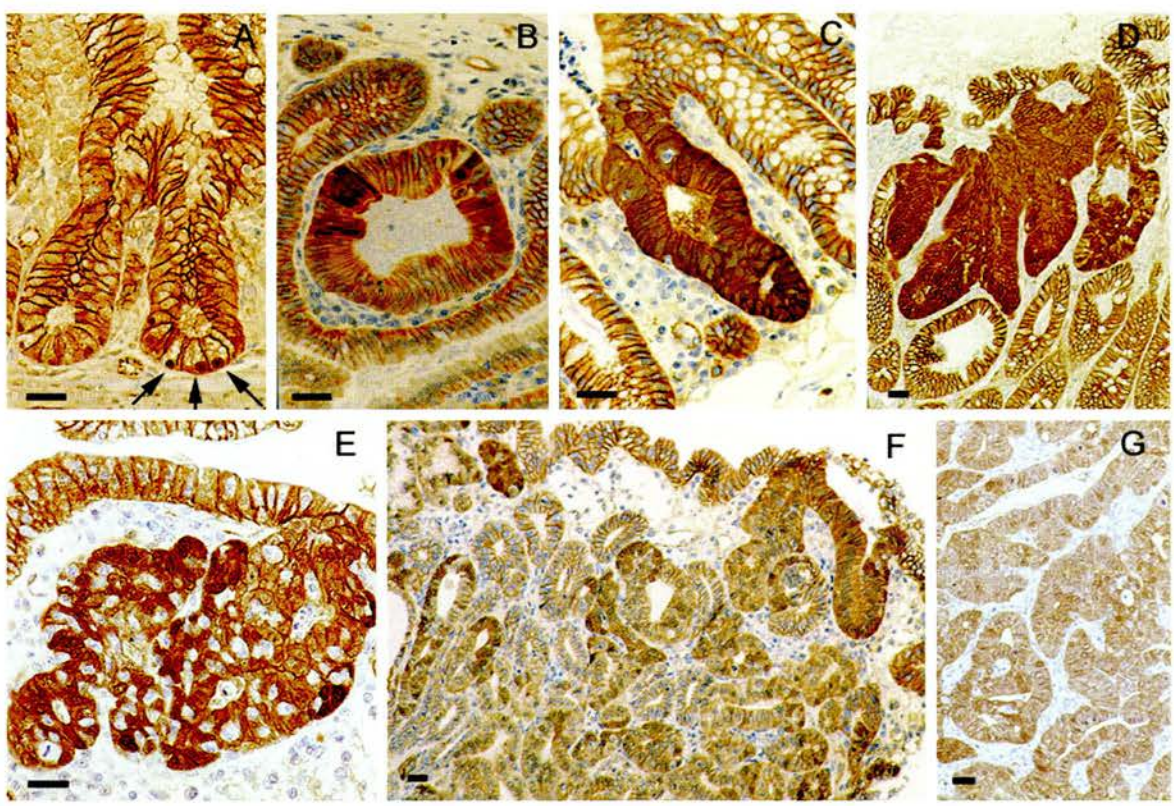

Figure 1 The pattern of β-catenin staining in the intestine of $Apc^{Min}+/-$ and ($Apc^{Min}+/-$, p53−/−) animals. Mice mutant for *Msh2* (De Wind *et al*., 1995), *p53* (Clarke *et al*., 1993) and Apc^{Min} (Moser *et al*., 1992) were maintained as outbred colonies segregating for Ola/129, Balb C, SWR and C57Bl/6 genomes. Mice were monitored on a daily basis for signs of ill health and were killed when they showed signs of disease. All tissues were paraffin embedded using standard methods after overnight fixation in either buffered formalin or methacarn (four parts methanol, two parts chloroform, one part acetic acid v/v). High temperature antigen retrieval was performed (Alman *et al*., 1997), sections were cooled to room temperature and immersed in 1.5%H_2O_2 solution to block endogenous peroxidase for 15 min. Sections were then incubated with 1 : 50 mouse monoclonal β-catenin antibody (IgG_1, clone 14, Transduction Laboratories, USA) for 60 min, and subsequently with 1 : 400 Rabbit Anti-Mouse Biotinylated secondary antibody (DAKO) for 30 min. The sections were incubated in StrepABComplex/HRP (DAKO) for 30 min. The labelled complex was developed with diaminobenzidine (DAB, 0.5 mg/ml) for 5–8 min. at room temperature. (**a**–**g**) Photographs demonstrating the various features observed in animals with these genotypes. All the features illustrated here were observed irrespective of *p53* status. All scale bars represent 10 μm (**a**) β-catenin staining in morphologically normal crypts of the small intestine. β-catenin was detected throughout the cytoplasm of epithelial cells but was strongly localized to the lateral borders. Strong nuclear localization was observed in cells at the crypt base (arrows). (**b**) Heterogeneous expression in a type I lesion. The majority of cells show the normal pattern of staining, with localization to the lateral borders. A subset of cells show increased cytoplasmic and nuclear staining. (**c**) Uniformly increased β-catenin staining within a type 1 lesion. (**d**) Increased β-catenin staining in a type II lesion. (**e**) Heterogeneous expression in a type II lesion. Cells showing increased β-catenin showed localized to the cytoplasm and in some instances localization to the nucleus. (**f**) Heterogeneous expression of β-catenin within a type III lesion. (**g**) Reduced expression within a type IV lesion. Where expression of β-catenin was retained this was often localized to the nucleus

In order to further characterize the association between tumourigenesis and dysregulation of β-catenin we have analysed the pattern of β-catenin expression in normal and neoplastic tissue derived from mice mutant for the tumour suppressor genes *p53* and *Apc*. This analysis is performed on mice which carry a mutant *Apc* allele. Loss of the remaining wild type *Apc* allele results in dysregulated expression of β-catenin. Because we also wished to address the possibility that dysregulation of β-catenin is not an obligate step in intestinal neoplasia, we also analysed mice deficient for the DNA mismatch repair gene *Msh2* (De Wind *et al*., 1998), a murine model of hereditary non-polyposis colorectal cancer (HNPCC). *Msh2*−/− mice develop lymphomas with a peak incidence at 2–3 months of age (De Wind *et al*., 1995; Reitmair *et al*., 1996). Of the 50% of *Msh2*−/− mice which survive beyond 6 months of age, 70% develop intestinal neoplasms (Reitmair *et al*., 1996). We report here the pattern of β-catenin expression in lesions arising in mice mutant for *Msh2*−/− and (*Msh2*−/−, Apc^{Min}+/−).

We first investigated the pattern of expression of β-catenin in intestinal lesions arising in Apc^{Min} and p53/Apc^{Min} mutants. In morphologically normal epithelium, β-catenin was localized at the cell membrane. Nuclear localization was observed in some cells: these were always located at the crypt base (Figure 1a). This observation suggests a role for β-catenin in the base of the crypt as β-catenin is thought to mediate transcriptional regulation within the nucleus, and indeed interaction with the transcription factor Lef-1 is known to promote nuclear localization of β-catenin (Huber *et al*., 1996).

In both Apc^{Min}+/− and (Apc^{Min}+/−, p53−/−) mice, expression of β-catenin is more intense in dysplastic crypts and small adenomas. To control for staining variability between sections, changes in the intensity of expression were always scored relative to normal epithelium within the same section. The lesions were subclassified as in Clarke *et al*. (1995): (i) single dysplastic crypts, showing nuclear pleomorphism and stratification; (ii) complex lesions, comprising several architecturally distorted crypts in the lamina propria with virtually normal overlying surface epithelium; (iii) small adenomas, identified by the overall disturbance of architecture including the surface and distinguished from the previous category on the basis of increased size and surface involvement; (iv) large adenomas, and (v) adenocarcinoma. The pattern of β-catenin staining, summarised in Figure 2, was essentially identical in Apc^{Min}+/− and (Apc^{Min}+/−, p53−/−) mice, with all features described below noted in both groups. A substantial proportion of all lesion types showed heterogeneous expression of β-catenin expression, even where only single crypts were involved (type I lesions, Figure 1b,e and 2). The term 'heterogeneous' is used here to describe lesions in which only a proportion of cells were characterized by increased expression. Although heterogeneous β-catenin was observed in all lesions types, the proportion of cells overexpressing β-catenin were at their highest in type I-III lesions (Figure 1c–d and 2). Mosaic type IV lesions showed the lowest proportion of cells staining positive for β-catenin (Figure 1f). Large areas of reduced staining were observed in some late stage lesions, including those categorized both as type IV and V (Figure 1g). In all categories of lesion the predominant pattern was of increased β-catenin staining within the nucleus, within the cytoplasm and also at the cell membrane. However a pattern of strong nuclear localization without concomitant cytoplasmic staining was also observed within some lesions as has been previously reported (Sheng *et al*., 1998).

These results show that high levels of β-catenin are present in the majority of intestinal lesions, presumably as a direct consequence of perturbation of the Wnt pathway. Furthermore, areas showing high levels of β-catenin included those composed of heterogeneous or single dysplastic crypts in the intestine, supporting the notion that dysregulated β-catenin expression is an extremely efficient marker of early neoplastic change in the murine intestine. Lower levels of expression were seen in focal areas within some larger adenomas and adenocarcinomas, suggesting that genotypic changes which lead to elevated β-catenin are only relevant to the early stages of neoplasia. This concept is supported by observations of localized areas of reduced or absent β-catenin expression within some adenomas; and also by studies of human tumorigenesis, where down-regulation of both β-catenin and E-cadherin has been reported in a range of carcinomas (Takayama *et al*., 1996).

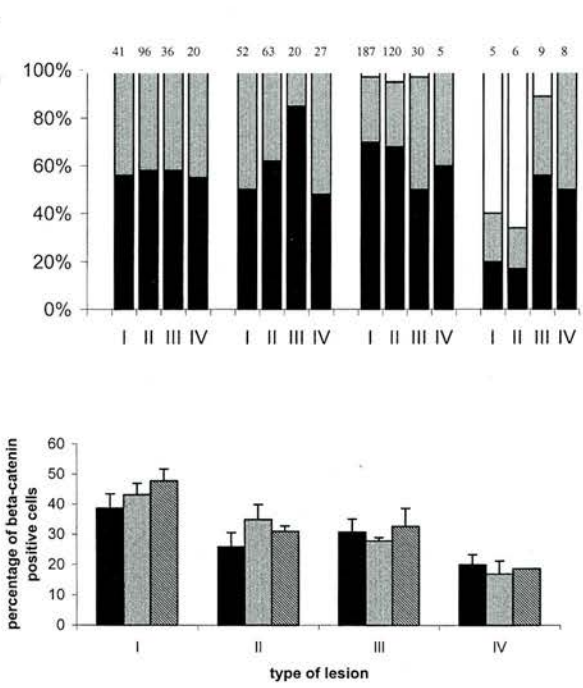

Figure 2 β-catenin expression patterns within each class of intestinal lesion. (**a**) Percentage of each lesion type showing either upregulation of β-catenin in all cells (black bars); a mosaic or heterogeneous pattern of upregulation as defined in the text (grey bars); or no upregulation (open bars). The number of lesions scored is shown over each column. Insufficient numbers of category V lesions were identified to permit scoring. (**b**) Histogram showing the percentage of cells expressing high levels of β-catenin within lesions characterized by mosaic expression of β-Catenin. Black bars, Apc^{Min}+/−; Grey bars (Apc^{Min}+/−, *p53*−/−); Hatched bars (Apc^{Min}+/−, *Msh2*−/−). Mean values are given for each lesion category, as defined in the text. Error bars represent SEM. Insufficient numbers of mosaic lesions were identified in *Msh2*−/− mice to permit analysis

We next investigated the pattern of expression of β-catenin in intestinal lesions arising in Msh2 mutant animals and Msh2/Apc^{Min} mutants (summarized in Figure 2). Previous studies have shown that the Msh2 mutation predisposes to intestinal tumorigenesis and also accelerates neoplasia in Apc^{Min}+/− mice (De Wind *et al*., 1995, 1998; Reitmair *et al*., 1996). In Msh2−/− animals we identified type I , II and III lesions which showed normal β-catenin expression (Figure 3b), a phenomenon we did not observe in Apc^{Min}+/−mice (Figure 3a). However, all type IV adenomas were characterized by increased levels of β-catenin expression. No type V lesions were identified. In (Msh2−/−, Apc^{Min}+/−) mice there was a significant increase in the frequency of adenomas, as has been previously reported (Reitmair *et al*., 1996). The majority of these lesions stained strongly for β-catenin (Figure 3c), however we again identified a small number of type I,II and III lesions (× 10%) with the pattern of β-catenin expression characteristic of normal cells (Figure 3d). All type IV lesions analysed showed altered β-catenin expression, with an almost identical pattern to that observed in Apc^{Min}+/−mice. No type V lesions were identified.

We therefore successfully identified small lesions in both *Msh2* and *Msh2*/*Apc*^{Min} mice which showed normal levels and distribution of β-catenin. These findings show that dysregulated β-catenin is not an obligate event in early lesion formation, and furthermore that *Msh2* deficiency predisposes to such apparent β-catenin-independent events. Thus, *Msh-2* deficiency may predispose to dysplasia through mutations in other components of the Wnt signalling pathway which do not affect β-catenin levels or indeed through mutations in other path-

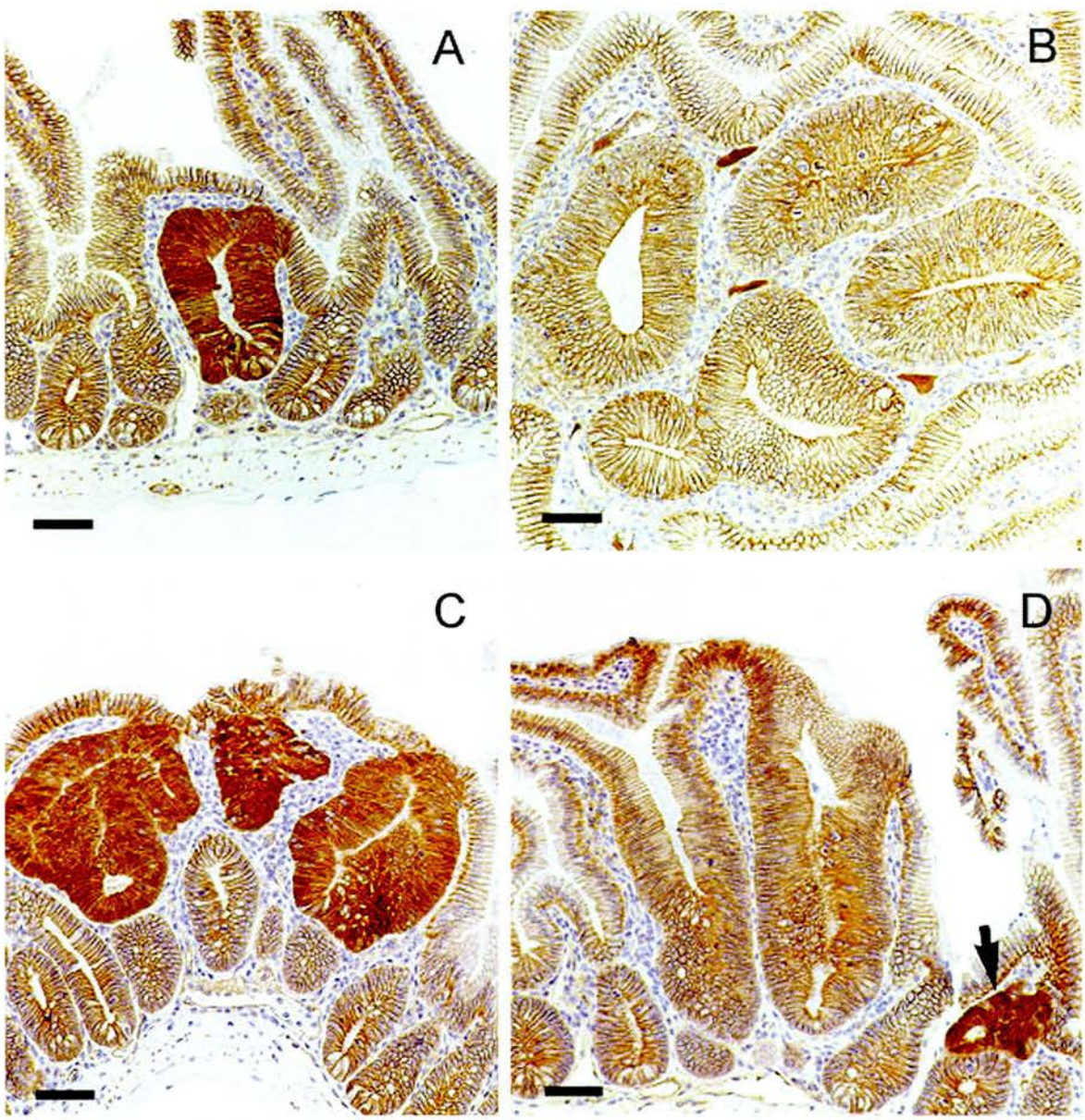

Figure 3 The pattern of β-catenin expression in the intestine of Msh2−/− mice (**a**,**b**) and (Msh2−/−, Apc^{Min}+/−) mice (**c**,**d**). Immunohistochemical analysis was performed as described in the legend to Figure 1. All scale bars represent 10 μm. (**a**) Increased β-catenin staining in a type I lesion. (**b**) Normal pattern of β-catenin expression in a type II lesion, with β-catenin strongly localized to the lateral borders. (**c**) Increased β-catenin expression in a type II lesion. (**d**) Normal β-catenin expression in a type II lesion, with retained localization to the lateral borders. A type I lesion showing β-catenin dysregulation is indicated for comparison (arrow)

ways. All large adenomas (type IV) were characterized by increased β-catenin, showing that this degree of morphological change is absolutely associated with events which dysregulate β-catenin levels.

We have previously described the phenotype of mice mutant for both *p53* and *Apc* (Clarke *et al*., 1995). In addition to intestinal lesions these mice develop pancreatic neoplasia, either adenoma or acinar adenocarcinoma, with almost 100% penetrance. The cell type observed in these lesions was predominantly acinar, although some foci showed ductal transdifferentiation. The involvement of *Apc* in the development of pancreatic lesions was confirmed by loss of the remaining wild type allele in adenocarcinomas (Clarke *et al*., 1995). We therefore next wished to assess the extent of dysregulation of β-catenin in these lesions.

Within morphologically normal pancreatic cells, β-catenin was observed at the cell membrane, with no obvious nuclear localization. In ($Apc^{Min}+/-$p53$-/-$) mice all foci showing histological change were characterized by high levels of β-catenin. Increased staining was also seen in foci which were virtually histologically normal. Such increased staining was never observed in pancreas samples derived from wild type mice. Foci varied in size, with some containing only single or a few cells in the plane of section (Figure 4a,b). The observation of few or single cell lesions strongly suggests that dysregulated expression occurs very early in neoplasia.

In lesions identified in formalin fixed tissues, increased β-catenin expression was seen within both the nucleus and cytoplasm (Figure 4c). However, cytoplasmic staining was rarely observed in Methacarn fixed sections, suggesting that the observed cytoplasmic localisation was an artefact of tissue fixation. Using either fixation protocol we observed rare (<1%) lesions which did not show increased nuclear staining (Figure 4d). Nuclear atypia was seen in the majority of lesions, although the extent of nuclear pleomorphism varied considerably from mild to severe within each lesion (Figure 4b,e). Acinar cell lesions classified as adenoma were also characterized by increased β-

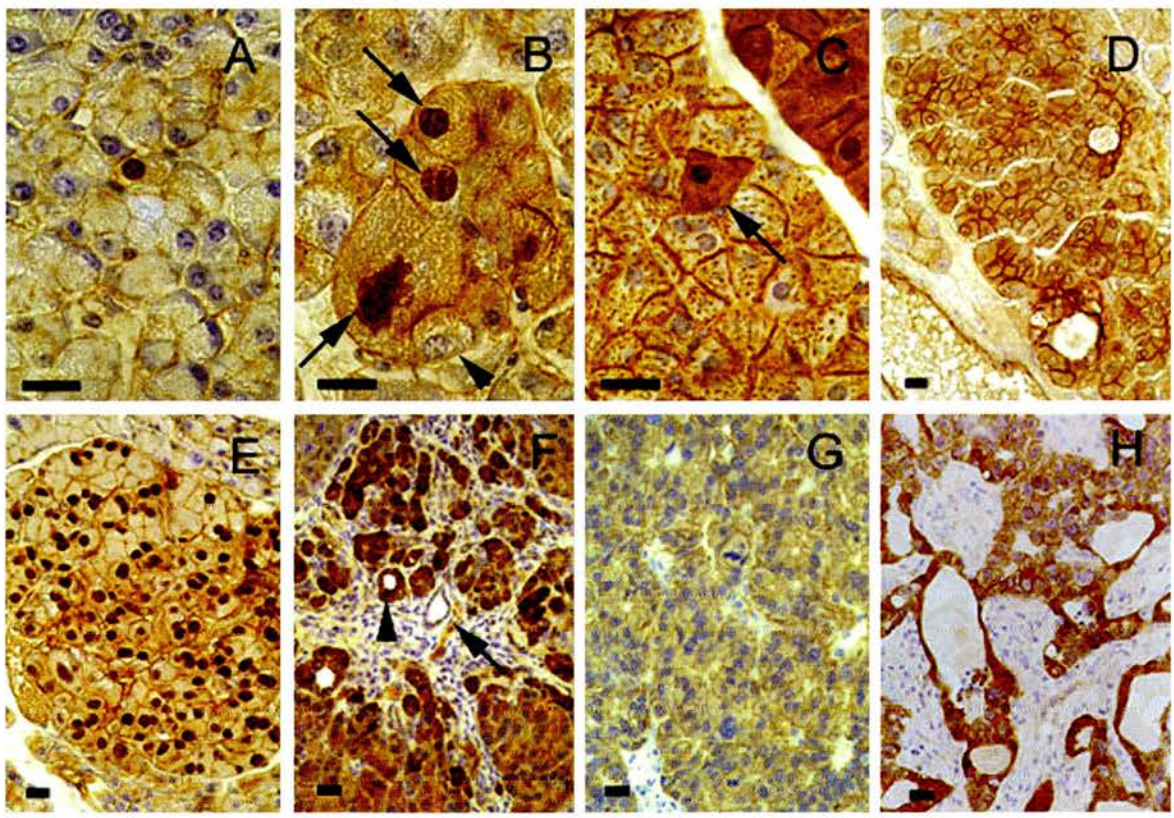

Figure 4 The pattern of β-catenin staining in the pancreas of $Apc^{Min}+/-$ and ($Apc^{Min}+/-$, p53$-/-$) mice. Immunohistochemical analysis was performed as described in the legend to Figure 1. (**a**–**e**) are representative of the patterns of β-catenin staining and histological atypia observed in the pancreas of both $Apc^{Min}+/-$mice and ($Apc^{Min}+/-$, *p53*$-/-$) mice. (**f**–**h**) are representative of these patterns in pancreatic adenomas and adenocarcinomas arising in ($Apc^{Min}+/-$, *p53*$-/-$) mice. All scale bars represent 10 μm. (**a**) Methacarn fixed. A single pancreatic acinar cell characterised by increased nuclear and cytoplasmic expression. The surrounding acinar cells are representative of the normal pattern of β-catenin staining, with localization to the cell borders. (**b**) Methacarn fixed. Small focus of acinar cells with increased expression. These foci were often composed of cells with increased nuclear size, prominent examples of which are indicated by arrows. This focus also contains a cell (short arrow) with no increase in nuclear levels of β-catenin. (**c**) This picture demonstrates the pattern of staining observed in formalin fixed tissues. Cells (arrowed or restricted to the upper right hand portion of this photograph) showing increased nuclear and cytoplasmic levels of β-catenin staining. (**d**) Methacarn fixed. A dysplastic adenoma showing increased β-catenin expression, but with no apparent nuclear localization. (**e**) Methacarn fixed. Increased β-catenin staining in a pancreatic focus, showing strong nuclear localization. Again, these foci were often composed of cells with increased nuclear size. (**f**) Methacarn fixed. Heterogeneous β-catenin expression in an adenoma containing areas of acinar-ductal transdifferentiation (arrowhead). No increase in β-catenin staining was detectable in normal ducts (arrow). (**g**) Low levels of β-catenin within an acinar adenocarcinoma. (**h**) Methacarn fixed. Areas of ductal differentiation within an acinar adenocarcinoma which have retained high levels of β-catenin expression

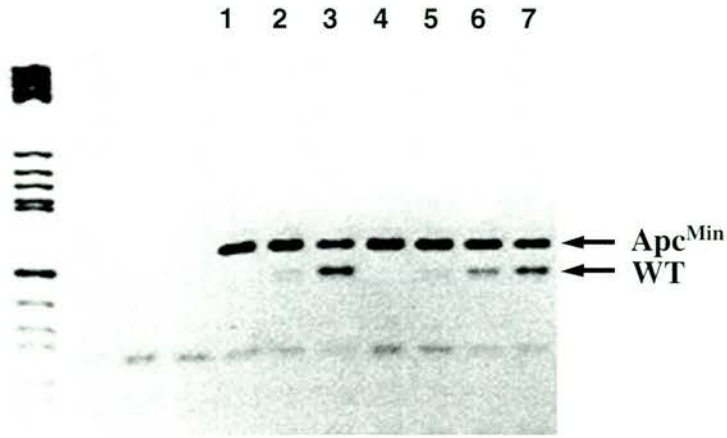

Figure 5 PCR amplification of Apc alleles from microdissected lesions. PCR analysis. Histological microdissection was performed as previously described (Going and Lamb, 1996). Samples were digested in proteinase K (1 mg/ml) and 1% Tween 20. The proteinase K was subsequently heat-inactivated at 95°C, for 10 min. PCR amplification was then performed essentially as previously described (Luongo *et al* 1994) using the primers (5′TCTCTT CTGAGAG CAGAAGTT) and (5′ATAGCCAA AGTTATGGAA GAAGTATCA). Representative results from PCR analysis of microdissected lesions. Determination of Min status was by PCR and *Hind*III digest of PCR product as previously described (Luongo *et al*., 1994). WT, the amplification product from the wild type *Apc* allele. Apc^{Min}, the amplification product from the mutant allele. Samples were all derived form lesions arising within Apc^{Min} mutant mice and were as follows: lanes 1 and 2, pancreatic foci showing β-catenin dysregulation; lane 3 normal pancreas; lanes 4 and 5, small intestinal lesions; lanes 6 and 7 normal intestinal epithelium. All results were obtained using microdissected areas containing a minimum of 50 cells

catenin staining. Some of these lesions contained areas of acinar-ductal transdifferentiation which were also strongly stained (Figure 4f). Adenocarcinomas contained areas in which β-catenin intensity was reduced (Figure 4g), however areas of ductal differentiation within these tumours retained high levels of β-catenin (Figure 4h).

These results prompted us to analyse Apc^{Min} mice. When these animals are maintained on a wild type background they do not develop pancreatic adenomas (Clarke *et al*., 1995). Surprisingly, although we confirmed absolute absence of neoplasms of the pancreas, we did find multiple foci of β-catenin dysregulation identical to those observed in (Apc^{Min} +/−p53−/−) mice. Our previous analysis (Clarke *et al*., 1995) had identified focal mild dysplasia in one out of seven *ApcMin* heterozygotes. Subsequent re-examination of these sections showed multiple ill-defined areas containing cells characterized by nuclear size variation. These areas overexpressed β-catenin. Thus, β-catenin immunohistochemistry efficiently highlighted focal areas of early histological change in the pancreas of both (Apc^{Min}+/−p53−/−) and Apc^{Min}+/−mice. We also analysed pancreatic tissue derived from *Msh2*−/−mice and (*Msh2*−/−, Apc^{Min}+/−) mice, neither of which develop spontaneous pancreatic neoplasms. No abnormal expression of β-catenin or histological atypia was observed in *Msh2*−/−mice. However, in (*Msh2*−/−, Apc^{Min}+/−) mice we again identified foci of β-catenin overexpression, and these did not differ in morphological appearance from those seen in Apc^{Min}+/−animals.

In the pancreas, dysregulated β-catenin expression was seen in 100% of lesions which appeared morphologically abnormal. Previously, we had noted the presence of these lesions at high frequency only in (*p53*−/−, Apc^{Min}+/−) mice and rarely in wild type mice. The occurrence of high β-catenin expression in areas of minimal histological abnormality in Apc^{Min} heterozygotes allows an order of genetic events to be proposed for this model of pancreatic neoplasia. In the presence of wild type *p53* such dysregulated expression does not lead to neoplasia, but it is associated with nuclear size variation, raising the possibility that loss of Apc function may promote chromosomal instability. We are currently characterizing this phenomenon in greater detail. By contrast, a *p53* null environment allows progression to adenoma and then adenocarcinoma. Loss of *p53* is therefore essential for adenoma formation in the time frame analysed here. Notably, the requirement for genetic change differs between pancreas and intestine, as within the murine intestine *p53* loss does not increase either adenoma burden or neoplastic progression (Clarke *et al*., 1995).

To characterize the status of the remaining *Apc* allele in both the intestinal and pancreatic lesions arising in Apc^{Min}+/− mice we performed PCR analysis on microdissected foci. Serial sections were generated and areas of increased β-catenin staining identified. These areas were microdissected and DNA isolated. Loss of the remaining Apc^{wt} allele was assessed following PCR amplification. This approach allowed us to analyse intestinal and pancreatic lesions of, at the lowest limit, approximately 50 cells per cross section. Using this method we demonstrate loss of the remaining wild type *Apc* allele in both the intestinal and pancreatic lesions analysed from Apc^{Min}+/− and Apc^{Min}+/−, *p53*−/− mice (Figure 5). This finding is consistent with the concept that β-catenin dysregulation occurs as a consequence of loss of Apc function.

Taken together, these results show that β-catenin dysregulation occurs in both the intestine and pancreas, and that where present it is associated with the very first steps in the development of neoplasia. These findings demonstrate that altered expression of β-catenin is a key marker of *Apc* dysregulation in both these tissues, and suggest that altered β-catenin expression may be a useful diagnostic marker of early

neoplastic change in human disease. However, we also show that β-catenin dysregulation is not an obligate step in the generation of intestinal lesions in an *Msh2* deficient background, and therefore that other mechanisms may underlie such early neoplastic change.

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